Diversity and properties of aerobic anoxygenic photosynthetic bacteria in epilithic biofilms of a river

A doctoral dissertation

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Abstract

Aerobic anoxygenic photosynthetic bacteria grow heterotrophically by respiration. They produce bacteriochlorophyll but do not grow anaerobically in the light. Aerobic anoxygenic photosynthetic bacteria were found to be a considerable fraction of marine bacterioplankton; however their distribution in freshwater environments has not been well-understood. Biofilm on stones under water is an important microbial habitat in the ecosystem of rivers. In this study, I explored aerobic anoxygenic photosynthetic bacteria in the streambed biofilms of the upper reaches of Tama River, Japan using culture-dependent and culture-independent approaches. The physiological properties of isolates were also characterized.

Total genomic DNAs were directly extracted from the biofilm collected in Ohme-city. Diversity of the PCR-amplified *pufM* gene coding for a subunit of the photochemical reaction center was analyzed. Thirty seven sequences were obtained and phyolgenetically divided into 18 operational taxonomic units (OTUs). Each OTU was defined as a group having amino acid sequence identities above 90%. One of the OTUs was closely related to anaerobic anoxygenic photosynthetic bacteria *Rhodoferax fermentans*. Using culture-dependent approach, isolates closely related to *R. fermentans* and anaerobic anoxygenic photosynthetic bacteria *Rhodopseudomonas palustris* were isolated from the same sample. Seventeen other OTUs showed less than 85 % identities to the PufM amino acid sequences of any known photosynthetic bacteria. Nine of them made clades with the sequences obtained from aerobic anoxygenic photosynthetic bacteria.

To isolate aerobic anoxygenic photosynthetic bacteria from the biofilms,

cultivation under aerobic conditions in the dark was conducted. Cells of biofilms collected from Ohme-city were suspended in sterilized distilled water and spread on agar plates. Spectroscopic analyses of the colonies indicated that about 20 % of the total number of colonies contained bacteriochlorophyll. Twenty eight strains were isolated and all isolates were confirmed to be aerobic anoxygenic photosynthetic bacteria because of the absence of growth under anaerobic photoheterotrophic conditions. Bacteriochlorophyll production was also confirmed for all the isolates. The bacteriochlorophyll contents varied by strains from 0.13 to 3.58 nmol per mg of dry cell weight. The isolated strains were phylogenetically divided into 8 groups belonged to alpha-1, alpha-3 and alpha-4 subclass of the class *Alphaproteobacteria* and the class *Betaproteobacteria* with 16S rRNA gene sequence similarities of 96 % or more. Among them, 4 groups were distantly related to the previously known bacteria.

The effect of organic nutrient concentrations on growth of the isolates was evaluated. Two strains grew in a low-nutrient medium (containing 0.5 g of organic compounds per liter) but did not grow in a high-nutrient medium (containing 2.5 g of organic compounds per liter). Other strains grew in both media.

Sampling was also conducted in Okutama-machi, 20 km upstream of the sampling site in Ohme-city. Aerobically grown randomly selected 13 strains of bacteriochlorophyll-containing bacteria were isolated. They were phylogenetically divided into 10 groups belonging alpha-1 and alpha-4 subclass of the class *Alphaproteobacteria* and the class *Betaproteobacteria*. Among them 5 groups were common to that in Ohme-city. Other than the groups obtained in Ohme-city, 4 additional groups which distantly related to the previously known bacteria were isolated.

Physiological properties of several phylogenetically and metabolically novel isolates were studied and compared to those of their neighbors. They showed different properties from their phylogenetic neighbors. Distinct temperature dependence was shown by the strains. They did not grow at a moderately high temperature at 40^{O}C. Some strains did not grow even at 35^{O}C.

This study showed phylogenetically divergent aerobic anoxygenic photosynthetic bacteria in addition to anaerobic anoxygenic photosynthetic bacteria inhabited biofilms of the river. Phylogenetic analyses indicated that 8 groups representing novel taxa severally were present in the isolates. It seems that the river biofilms contain a variety of habitats for aerobic anoxygenic photosynthetic bacteria as well as anaerobic ones. Differences in growth properties and bacteriochlorophyll contents may reflect environmental diversity of the habitats.

CONTENTS

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General Introduction

Phylogenetic positions of photosynthetic bacteria

Photosynthetic bacteria (phototrophic bacteria) comprise several independent phyla in bacterial phylogenetic tree, i.e., filamentous anoxygenic photosynthetic bacteria (*Chloroflexi*), green sulfur bacteria (*Chlorobi*), *Cyanobacteria*, heliobacteria (*Firmicutes*) and purple bacteria (*Proteobacteria*) (10). Photosynthetic bacteria were also recently found in the phyla *Acidobacteria* (3) and *Gemmatimonadetes* (22).

Among them, bacterial groups other than *Cyanobacteria* conduct anoxygenic photosynthesis, therefore they are called to be anoxygenic photosynthetic bacteria (anoxygenic phototrophic bacteria), whereas, *Cyanobacteria* conduct oxygenic photosynthesis and are called to be oxygenic photosynthetic bacteria (oxygenic phototrophic bacteria).

Energy metabolism of anoxygenic photosynthetic bacteria

Anoxygenic photosynthetic bacteria make bacteriochlorophyll as a photosynthetic pigment. Anoxygenic photosynthetic bacteria such as purple nonsulfur bacteria and filamentous anoxygenic photosynthetic bacteria are facultative anaerobes and use organic compounds as carbon and energy sources. They grow under anaerobic conditions in the light (photoheterotrophic growth) and aerobic conditions in the dark (chemoheterotrophic growth).

Aerobic anoxygenic photosynthetic bacteria

Anoxygenic photosynthetic bacteria had been known to conduct photosynthesis under anaerobic conditions. In 1978, bacteriochlorophyll-containing bacteria which grow only under aerobic conditions were reported at the first time (4, 13). As they do not grow under anaerobic conditions even in light, they were called aerobic anoxygenic photosynthetic bacteria (aerobic anoxygenic phototrophic bacteria) (14). Until now, more than 60 species of aerobic anoxygenic photosynthetic bacteria have been described in *Alpha-*, *Beta-* and *Gammaproteobacteria* from various environments (21).

Phylogenetically, aerobic anoxygenic photosynthetic bacteria and anaerobic purple photosynthetic bacteria are related to each other (11). Photosynthetic genes and consequently photosynthetic apparatus of both bacteria are closely similar. *Proteobacteria* are thought to have diverged from a photosynthetic ancestor (19). Aerobic anoxygenic photosynthetic bacteria and anaerobic purple photosynthetic bacteria are direct descendants of the ancestor (2).

Most of aerobic anoxygenic photosynthetic bacteria have significantly lower amounts of bacteriochlorophyll than anaerobic purple photosynthetic bacteria (5, 14), but some species have similar amounts of bacteriochlorophyll to those of the anaerobic ones (14).

Phylogeny of aerobic anoxygenic photosynthetic bacteria

Phylogenetically, aerobic anoxygenic photosynthetic bacteria have wide distribution in the phylum *Proteobacteria*. The majority of these bacteria has been found in the class *Alphaproteobacteria* (21).

Both members of aerobic anoxygenic photosynthetic bacteria and anaerobic

anoxygenic photosynthetic bacteria have been reported in the alpha-1, alpha-2 and alpha-3 subclass of *Alphaproteobacteria*, however in the alpha-4 subclass, only members of aerobic anoxygneic photosynthetic bacteria were found and no anaerobic anoxygnic photosynthetic species was contained in the subclass. All the aerobic anoxygenic photosynthetic members those belong to the alpha-3 subclass of *Alphaproteobacteria* were derived from saline or oceanic environments and they all belonged to the *Roseobacter* clade in the alpha-3 subclass of the class *Alphaproteobacteria* (20).

Anoxygneic photosynthetic bacteria have been rarely found in the class *Betaproteobacteria*. *Roseateles depolymerans* (16) was the first reported aerobic anoxygneic photosynthetic member in *Betaproteobacteria*. Next to this discovery, *Aquincola tertiaricarbonis* (9, 12) have been described as aerobic anoxygneic photosynthetic bacteria in the class *Betaproteobacteria*. *Methyloversatilis universalis* (6) which belongs to *Betaproteobacteria* have been also shown to have genes coding subunits of photosynthetic reaction center. Several strains of aerobic anoxygneic photosynthetic bacteria belonging to the class *Gammaproteobacteria*, such as marine bacterium *Congregibacter litoralis* (15) have been known.

Recently, an aerobic anoxygenic photosynthetic member was also found in the phylum *Gemmatimonadetes* (22).

Ecological importance of aerobic anoxygenic photosynthetic bacteria

Kolber *et al.* reported that aerobic anoxygenic bacterial photosynthesis is widespread and accounts for 2-5% of the photosynthetic electron transport in the upper ocean (6). They also reported that photosynthetically competent anoxygenic bacteria are

abundant in the upper open ocean and comprise at least 11% of the total microbial community (7). After these reports, many intensive studies have revealed the presence, abundance and diversity of aerobic anoxygenic photosynthetic bacteria in various regions of the oceans.

Biofilm on submerged stones of rivers (epilithic biofilm)

In upper reaches of rivers, primary production is mainly conducted in biofilms on submerged stones (1). This benthic microbial biofilms attached to rocks are called ‘epilithic biofilms’. The biofilms are important to sustain river ecosystems, where algae and cyanobacteria produce organic compounds and heterotrophs degrade them.

Aerobic anoxygenic photosynthetic bacteria in rivers

Taxonomy and ecology of aerobic anoxygenic photsynthetic bacteria in the freshwater environments have been poorly documented. Especially in riverine environments, only a few studies have been conducted (12, 13, 14).

The aim of this study

The aim of this study is to know the diversity and properties of aerobic anoxygneic photosynthetic bacteria in biofilms on stones of upper reaches of a river.

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CHAPTER I

Diversity of Purple Photosynthetic Bacteria, Inferred from *pufM* Gene, within Epilithic Biofilm in Tama River in Ohme-city, Japan

Abstract

The diversity of purple photosynthetic bacteria in algae-dominated biofilm of streambed in Tama River, Japan was investigated. Clone library analysis of the *pufM* gene encoding a subunit of the photochemical reaction center of purple bacteria detected 18 operational taxonomic units (OTUs) in several classes of *Proteobacteria*. Most of the OTUs showed less than 85% identities to the PufM amino acid sequences of known photosynthetic bacteria. These results suggest that phylogenetically divergent and unknown purple photosynthetic bacteria are present in the epilithic biofilm of the river.

Introduction

Purple photosynthetic bacteria have been isolated from a variety of environments, such as sediments, soils and waters in ponds, lakes, lagoons and ocean (10, 11). As purple bacteria are metabolically versatile, *e.g.*, photosynthesis, degradation of organic compounds, nitrogen fixation or sulfide oxidation, they play important roles in ecosystems in the light.

Microbial ecological studies on freshwater environments have found several groups of purple photosynthetic bacteria belonging to *Alpha-* or *Betaproteobacteria* from lakes (12, 17) and rivers (8, 9, 20, 23). Some of these purple bacteria have been known to be anaerobic anoxygenic phototrophs. The presence of aerobic anoxygenic phototrophs (AAP), which carry out photosynthetic reactions only under aerobic conditions, has been also indicated by phylogenetic or physiological studies. However, the distribution and ecophysiology of purple photosynthetic bacteria in freshwater environments has not been documented yet.

Epilithic biofilm is important to sustain the ecosystem in freshwater environments in terms of producing organic substrates, feeding animals and degrading organic matters. The streambed biofilm is known to be mainly composed of oxygenic phototrophs, *i.e.*, cyanobacteria and algae (2). However, there is no study targeting purple photosynthetic bacterial diversity in river biofilm. Dense assemblages of bacterial cells and their active respiration in biofilm possibly develop some anaerobic niches (6) even when phototrophs emit oxygen, and consequently both aerobic and anaerobic anoxygernic phototrophs may found their niches within river biofilm.

In this study, I applied a culture-dependent method under anaerobic conditions

and a molecular method based upon *pufM* gene encoding a subunit of the photochemical reaction center to investigate diversity of purple photosynthetic bacteria in epilithic biofilm at an upstream region of a river where the amount of dissolved organic matter is limited. Phylogenies based on *pufM* gene sequences are mostly consistent with those based on 16S rRNA gene (14), and thus the *pufM* gene is frequently utilized for genetic surveys of anoxygenic phototrophs (1, 4, 12, 16, 24).

Materials and Methods

Submerged cobbles of about 15 to 25 cm in the longest length were collected from streambed in river located in the upstream region of Tama River in Ohme-city (35°47′13″N, 139°15′15″E), in the west suburbs of Tokyo, Japan in August 2009. The river width at the sampling site was 40 m. The water depth of the sampling site was about 20 cm. Water temperature, pH, biochemical oxygen demand (BOD) and flow velocity of the river water at the sampling time were 18 °C, 7.6, 0.5 mg L^{-1} and 0.4 m s^{-1}, respectively. Average values of dissolved oxygen, total nitrogen and total phosphorus in this region in July to September 2009 were 8.9 ± 0.3 mg L^{-1}, 0.79 ± 0.10 mg L^{-1} and 0.017 ± 0.005 mg L^{-1}, respectively (a monthly report by Bureau of Environment, Tokyo metropolitan government, http://www.kankyo.metro.tokyo.jp). A brownish biofilm of about 1 mm thickness was developed on the cobbles. A total 150 cm^2 area of the epilithic biofilm was scraped off from the top surface of the each cobble using a sterile toothbrush and suspended into 10 mL of sterile distilled water.

For bacterial culture, 0.1mL of the biofilm suspension was transferred into a 30 mL volume of screw cap tube filled with a PE medium (7), a semisynthetic medium containing organic compounds. The tubes were incubated at 30°C under the filtered incandescent light (ca. 2,000 lux) of wavelength over 700 nm for 7 to 14 days. The cultures which showed spectral properties of purple bacteria were streaked on agar plates of the PE medium. The plates were incubated anaerobically under the incandescent light and red-colored colonies were transferred and streaked on new plates. These operations were repeated more than twice to obtain pure cultures. These isolation procedures were conducted using the samples obtained in August 2009 as well as those

obtained in May 2009.

Total genomic DNAs were directly extracted from the collected biofilm according to Noll *et al.* (15). DNA fragments of *pufM* gene coding for the M subunit of the photochemical reaction center were amplified. Nested PCR was conducted to amplify *pufM* gene fragments from environmental DNA using primer sets pufLM-F/pufLM-R (1st PCR) (14) and M150f/M572r (2nd PCR) (16). PCR products (approximately 380 bp) were cloned with the pTAC-1 Vector (DynaExpress TA cloning kit, BioDynamics Laboratory, Tokyo, Japan). *Escherichia coli* JM109 competent cells (Nippon gene, Tokyo, Japan) were transformed according to manufacturer's instructions. DNA sequences were determined with the BigDye v3.1 cycle sequencing kit (Applied Biosystems, Foster city, CA, USA) and a DNA sequencer ABI3130xl (Applied Biosystems). Chimeric clones were checked manually and excluded from further analyses. The phylogenetic tree based on the amino acid sequences of the partial PufM was constructed using the neighbor-joining and maximum-likelihood methods with a MEGA version 5 program (19, 22).

The nucleotide sequences determined in this study have been deposited in the GenBank/EMBL/DDBJ database under accession numbers AB670200 to AB670233.

Results

Cultivation of the river biofilm on the agar plates of PE medium yielded red-brown colonies. All of these colonies were similar in morphology to each other. Among them, 17 strains were isolated to determine the *pufM* sequences. The sequence analysis indicated that there were 5 different isolates, two of which were designated Tisolate 25 and Tisolate 231 in Fig. I-1 since these two isolates had been obtained from the sample used for the direct extraction of total genomic DNA and subsequent clone library analysis. The closest relatives of these isolates are listed in Table I-1.

Fig. I-1 shows a neighbor-joining tree based on the amino acid sequences inferred from the partial *pufM* gene sequences from 37 clones and two isolates obtained in this study together with those from database. A phylogenetic tree using the maximum-likelihood method (figure not shown) showed a tree topology roughly consistent with that in Fig. I-1. Two major clades were recognized; one containing alpha-1, alpha-2, alpha-4 subclasses, beta and gamma classes of *Proteobacteria* and the other containing alpha-3 subclass of *Proteobacteria*. This was roughly in agreement with earlier studies concerning *pufLM* or *pufM* phylogeny (4, 14). Obtained clones were grouped into 18 operational taxonomic units (OTUs). Each OTU was defined as a group having amino acid sequence identities above 90%. These OTUs were widely distributed in the phylogenetic tree and many of them were distantly related each other. No dominant OTUs in terms of numbers of clones were found, since every OTU consisted of less than 5 clones.

OTUs except OTU 6 showed less than 85% identities to the PufM sequences of the cultivated bacteria in the database. Sequences of OTU 6, which has the same

sequence as that of Tisolate 25, closely related to that of *Rhodoferax fermentans* (accession no. D50650, 98.4% identity). The other isolate, Tisolate 231, had 100% PufM sequence identity to *Rhodopseudomonas palustris* (accession no. AB015977). *Rhodoferax fermentans* and *R. palustris* are known to be anaerobic anoxygenic phototrophs (10). Tisolate 25 and Tisolate 231 were grown photoheterotrophically under anaerobic conditions.

The alpha-3 clade contains 6 OTUs (OTUs 13 to 18). OTU 13 is distantly related to other members in this clade. Sequences included in OTU 14 showed very low identities to those of the database; the highest identity was 65.7% to that of an environmental clone (accession no. AY912082) (23) collected from river water. OTUs 15 to 18 formed a clade with *Staleya guttiformis* (now known as *Sulfitobacter guttiformis*) and alpha proteobacterium R2A163 and R2A84 (21), those were reported as aerobic anoxygenic photosynthetic bacteria isolated from saline environments.

OTUs 1 and 2 were related to *Rhodospirillum rubrum*, belonging to the alpha-1 subclass of *Proteobacteria*, with 75.4% and 71.4% sequence identities, respectively. OTU 3 was found to be similar with a marine gamma proteobacterium (5) with sequence identity 84.1%. OTU4 was related to *Methylocella* sp. and *Rhodoplanes* sp. belonging to alpha-2 subclass of *Proteobacteria*. OTU 5 was similar to *R. fermentans* with identity 73.0%. It is indicated that OTUs 7 to 11 were grouped with the genus *Sphingomonas*, *Citromicrobium*, *Erythorobacter* and *Porphyrobacter* belonging to alpha-4 subclass. Photosynthetic bacteria in this subclass have been known to be aerobic anoxygenic phototrophs. OTU 12 showed low identities to known sequences, and the close relative was *Halorhodospira halophila*, belonging to *Gammaproteobacteria* (69.0% identity).

Discussion

In this study, I investigated diversity of purple photosynthetic bacteria in a streambed biofilm. Co-occurrence of possibly aerobic (*e.g.*, OTUs 15 to 18) and anaerobic (*e.g.*, OTU 6 and Tisolate 231) anoxygenic phototrophs was observed within the river biofilm as expected. Most OTUs detected by *pufM* clone library analysis had low identities to the sequences of cultured bacteria. Studies on bacterial communities in river biofilms using 16S rRNA gene analyses have also detected many clones of uncultured bacteria (3, 8, 13).

Phylogenetic analysis based on the PufM sequences indicated that purple photosynthetic bacteria in the river biofilm are widely distributed to alpha subclasses, beta and gamma classes of *Proteobacteria*. Such a high diversity of purple photosynthetic bacteria has never been reported in other environments. In French Mediterranean coast lagoon sediments, clones with *pufM* of the alpha-3 subclass of *Proteobacteria* accounted for 94.9 % of total clones (18). In the case of Antarctic lake water, no clones were related to alpha-3 subclass of *Proteobacteria* but 80 % of the clone contained *pufM* of the related taxa to *Betaproteobacteria* (12). Microenvironments of the algae-dominated river biofilm under the shallow and rapid flow of water would be highly heterogeneous, differing in concentrations of dissolved oxygen, organic and inorganic compounds. The population of purple photosynthetic bacteria within the epilithic biofilm microflora may be low because nested approach was required to amplify their DNAs. The niche for purple photosynthetic bacteria in the biofilm may be restricted, but their niches seem to be largely diverged.

As purple photosynthetic bacteria have bacteriochlorophylls which have

absorption bands at the different wavelengths from those of chlorophylls in oxygenic phototrophs, purple photosynthetic bacteria can capture the light energy even in the algae-dominated biofilms of rivers. In the biofilm community, in addition to the primary production and the degradation of the organic matters, some purple photosynthetic bacteria possibly contribute to oxidize sulfide produced by sulfate reducing bacteria, since I observed sulfide production from anaerobic culture of the epilithic biofilm used in this study when illumination was stopped (data not shown).

This study demonstrated an unexpected diversity of purple photosynthetic bacteria in the river biofilm. Physiological studies of the yet-to-be cultured epilithic purple photosynthetic bacteria will clarify the roles of these bacteria in the ecosystem of river.

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Table Ⅰ-1 Anaerobic anoxygenic purple photosynthetic bacteria isolated from the sampling site

Strain name	16S rRNA gene Closest relative (identity %)	*pufM* gene Closest relative (identity %)	Affiliation	Sampling date	Sample
isolate 25	*Rhodoferax fermentans* (98)	*Rhodoferax fermentans* (96)	β	August 25 2009	river biofilm
isolate 231	*Rhodopseudomonas palustris* (99)	*Rhodopseudomonas palustris* (100)	α-2	August 25 2009	river biofilm
isolate 51	*Rhodobacter capsulatus* (94)	*Rhodobacter capsulatus* (99)	β	August 25 2009	apical portion of filamentous algae streamer on a river submerged stone
isolate 322	*Rubrivivax gelatinosus* (99)	*Rubrivivax gelatinosus* (89)	α-3	May 21 2009	bottm portion of filamentous algae streamer on a river submerged stone
isolate 63	*Rhodoplanes elegans* (99)	not amplified	α-2	May 18 2009	river water

α-2, α-3 and β in Affiliation culumn indicate alpha-2, alpha-3 classes of *Alphaproteobacteria* and *Betaproteobacteria, plane*.

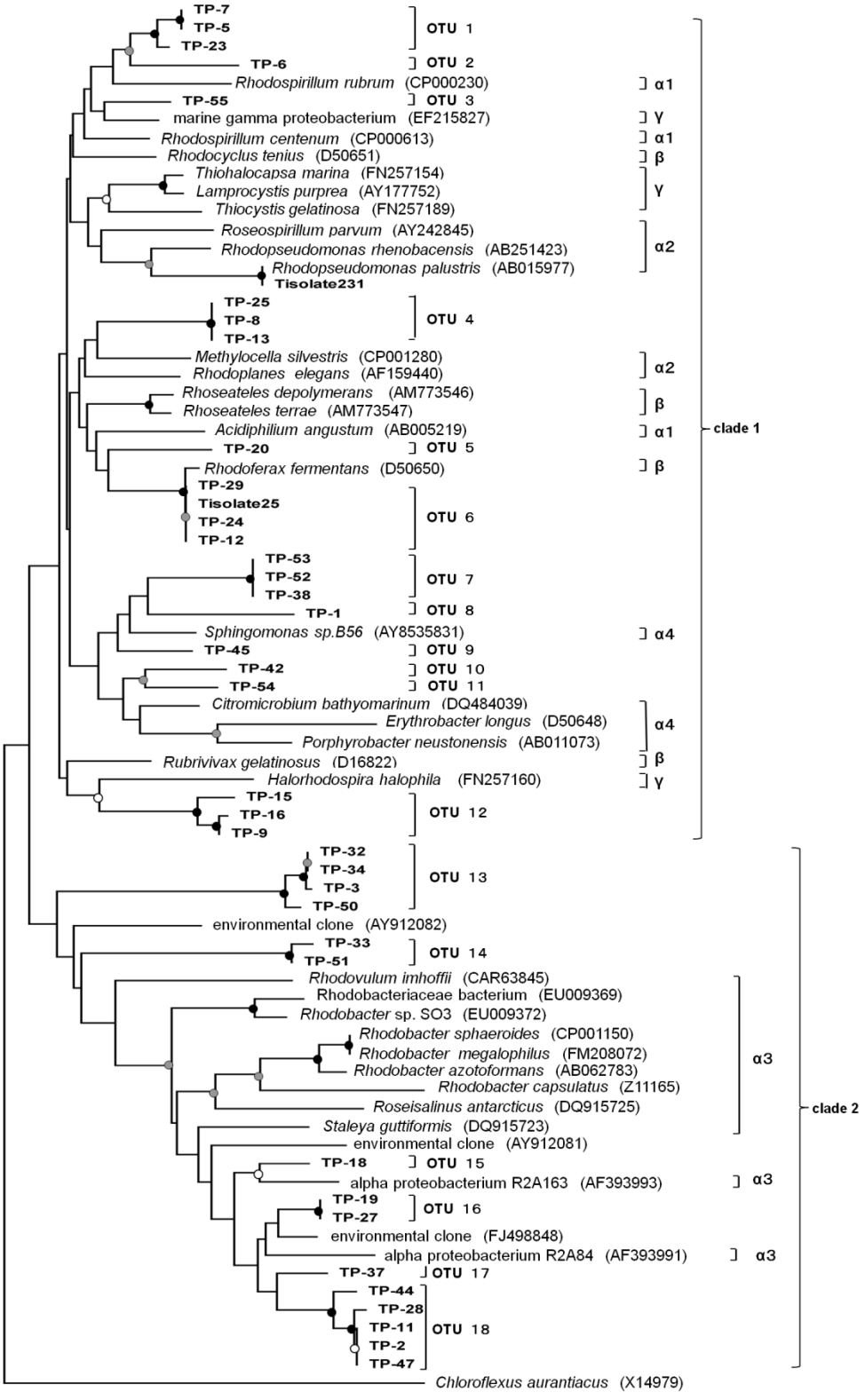
TP-7
TP-5
TP-23
OTU 1
TP-6
OTU 2
Rhodospirillum rubrum (CP000230)
α1
TP-55
OTU 3
marine gamma proteobacterium (EF215827)
γ
Rhodospirillum centenum (CP000613)
α1
Rhodocyclus tenius (D50651)
β
Thiohalocapsa marina (FN257154)
Lamprocystis purprea (AY177752)
Thiocystis gelatinosa (FN257189)
γ
Roseospirillum parvum (AY242845)
Rhodopseudomonas rhenobacensis (AB251423)
Rhodopseudomonas palustris (AB015977)
Tisolate231
α2
TP-25
TP-8
TP-13
OTU 4
Methylocella silvestris (CP001280)
Rhodoplanes elegans (AF159440)
α2
Rhoseateles depolymerans (AM773546)
Rhoseateles terrae (AM773547)
β
Acidiphilium angustum (AB005219)
α1
TP-20
OTU 5
Rhodoferax fermentans (D50650)
β
TP-29
Tisolate25
TP-24
TP-12
OTU 6
TP-53
TP-52
TP-38
OTU 7
TP-1
OTU 8
Sphingomonas sp.B56 (AY8535831)
α4
TP-45
OTU 9
TP-42
OTU 10
TP-54
OTU 11
Citromicrobium bathyomarinum (DQ484039)
Erythrobacter longus (D50648)
Porphyrobacter neustonensis (AB011073)
α4
Rubrivivax gelatinosus (D16822)
β
Halorhodospira halophila (FN257160)
γ
TP-15
TP-16
TP-9
OTU 12
clade 1
TP-32
TP-34
TP-3
TP-50
OTU 13
environmental clone (AY912082)
TP-33
TP-51
OTU 14
Rhodovulum imhoffii (CAR63845)
Rhodobacteriaceae bacterium (EU009369)
Rhodobacter sp. SO3 (EU009372)
Rhodobacter sphaeroides (CP001150)
Rhodobacter megalophilus (FM208072)
Rhodobacter azotoformans (AB062783)
Rhodobacter capsulatus (Z11165)
Roseisalinus antarcticus (DQ915725)
Staleya guttiformis (DQ915723)
α3
environmental clone (AY912081)
TP-18
OTU 15
alpha proteobacterium R2A163 (AF393993)
α3
TP-19
TP-27
OTU 16
environmental clone (FJ498848)
alpha proteobacterium R2A84 (AF393991)
α3
TP-37
OTU 17
TP-44
TP-28
TP-11
TP-2
TP-47
OTU 18
clade 2
Chloroflexus aurantiacus (X14979)
0.1

Fig. I-1.

Fig. I-1. Phylogenetic tree of *Proteobacteria* based on partial PufM amino acid sequences inferred from gene sequences. *Chloroflexus aurantiacus* was used as an outgroup. PufM amino acid sequences from environmental DNA in this study are indicated by TP-1–TP-55, and those from isolates in this study are indicated by the 'Tisolate' prefix. Sequences from the database are represented with their respective accession numbers after bacterial names in parentheses. OTUs are indicated to the right of the tree. Alpha-1, alpha-2, alpha-3, alpha-4 subclass, beta and gamma class of *Proteobacteria* are also indicated by α1, α2, α3, α4, β and γ to the right of the tree. Bootstrap values >90, 70-89 and 50-69% are indicated by black, gray and open circles, respectively. Scale bar represents the number of substitutions per site.

CHAPTER II

Phylogentically Diverse and Novel Aerobic Anoxygenic Photosynthetic Bacteria were Isolated from Epilithic Biofilms in Tama River in Ohme-city, Japan

Abstract

The ecological importance of aerobic anoxygenic photosynthetic bacteria in ocean environments has recently become evident but less is known about them in freshwater environments. To grasp the diversity and physiology of aerobic anoxygenic photosynthetic bacteria in river biofilm, the colony formation method was applied. Biofilm suspensions were spread on agar plates and cultivated aerobically in the dark. Spectroscopic analysis of the colonies indicated significant ratio of the total colonies contained bacteriochlorophyll. Twenty eight isolated strains were confirmed to be aerobic anoxygneic photosynthetic bacteria by the absence of growth under anaerobic photosynthetic conditions. Bacteriochlorophyll production was also confirmed for all the isolates, and the bacteriochlorophyll content varied by strain from 0.13 to 3.58 nmol per mg of dry cell weight. The isolated strains were phylogenetically divided into 8 groups, having 96% to 100% 16S rRNA sequence identities within each group, belonging to the alpha-1, alpha-3 and alpha-4 subclasses of *Alphaproteobacteria* and *Betaproteobacteria.* Among them 4 groups were distantly related to previously known bacteria with similarities of 97.3-98.1%. Two strains grew in a low-nutrient medium containing 0.5 g of organic compounds per liter but did not grow in a high-nutrient medium containing 2.5 g of organic compounds per liter. These results suggest occurrence of diverse and unknown aerobic anoxygenic photosynthetic bacteria in river biofilm.

Introduction

Aerobic anoxygenic photosynthetic bacteria grow heterotrophically by aerobic respiration. They produce bacteriochlorophyll but do not grow anaerobically in the light (21). Imhoff and Hiraishi explained that aerobic anoxygenic photosynthetic bacteria are thought to use photosynthesis as a supplementary energy source (9).

Since first report in 1978 (7, 20), more than 60 species of aerobic anoxygenic photosynthetic bacteria have been described in *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* (29). This group was recently recognized to be widespread in the ocean, account for a considerable fraction, for example 11% of the marine bacterioplankton, and reported to have significant roles for marine carbon cycling (12, 13). However, presence and diversity of aerobic anoxygenic photosynthetic bacteria in freshwater environments have been poorly understood.

I had reported the diversity of photochemical reaction center (*puf*) gene for anoxygenic photosynthesis detected from river biofilm (8, Chapter I), suggesting the existence of several novel lineages of anoxygenic photosynthetic bacteria in *Proteobacteria*. Some of them are suggested to be aerobic anoxygenic photosynthetic bacteria on the basis of the phylogenetic analysis based on *puf* gene sequences. However, physiological analyses are necessary to confirm them as aerobic anoxygenic photosynthetic bacteria. In this study, I isolated bacteriochlorophyll-containing bacteria from epilithic biofilms of upper reaches of Tama River, Japan and phylogenetically and physiologically characterized the isolates as aerobic anoxygenic photosynthetic bacteria.

Materials and Methods

Sampling of epilithic biofilms

Submerged cobbles of about 15 to 20 cm in the longest length were collected from a streambed in a sunny river located in the upper reaches of Tama River in Ohme City (35°47'13"N, 139°15'15"E, Altitude is 200 m), Tokyo, Japan on February 20 and July 23, 2012. Biofilm of about 1 mm thickness was present on the cobbles. The river width and water depth at the sampling site were about 40 m and 0.2 m, respectively. Water temperature, pH and flow velocity of the river water at the sampling time were 3.0°C, 8.0, 0.6 to 0.9 m s^{-1}, respectively, in February and 13.8°C, 8.2, 0.3 to 1.2 m s^{-1}, respectively, in July. Average values of biochemical oxygen demand, dissolved oxygen, total nitrogen and total phosphorus of the river water in this region were 0.26±0.28 mg L^{-1}, 11.8±0.73 mg L^{-1}, 0.58±0.05 mg L^{-1}, 0.013±0.014 mg L^{-1}, respectively, in February 2012 and 0.73±0.17 mg L^{-1}, 9.6±0.21 mg L^{-1}, 0.75±0.04 mg L^{-1}, 0.009±0.003 mg L^{-1}, respectively, in July 2012 (monthly report by Bureau of Environment, Tokyo Metropolitan Government, http://www.kankyo.metro.tokyo.jp). A total 150 cm^2 area of epilithic biofilm was scraped off from the top surface of each cobble using a sterilized toothbrush and suspended in 10 ml of sterilized distilled water. Samples were taken from 3 cobbles within 10 m^2 area. The suspensions were kept cool in ice and brought to the laboratory.

Isolation of bacteriochlorophyll-containing aerobic bacteria

Aggregation in the biofilm suspensions was dispersed using POLYTRON PT10/35 (KINEMATICA, Luzern, Switzerland) in a chilled bowl. After that, the

suspensions were serially diluted and spread onto agar plates. Gellan gum was also used instead of agar. Isolation was conducted using 2 types of media, PE medium (6) and Nutrient agar (Eiken Chemical, Tochigi, Japan). Composition of PE medium was 0.5 g of sodium glutamate, 0.5 g of sodium succinate, 0.5 g of sodium acetate, 0.5 g of yeast extract (Wako, Osaka, Japan), 0.5 g of casamino acids (Wako, Osaka, Japan), 0.5 g of sodium thiosulfate, 0.5 g of $(NH_4)_2SO_4$, 0.38 g of KH_2PO_4, 0.39 g of K_2HPO_4, 5 ml of a basal salt solution and 1ml of vitamin mixture per liter. The pH of the medium was adjusted to 7.5. The basal salt solution was composed of 1.11 g of $FeSO_4 \cdot 7H_2O$, 24.65 g of $MgSO_4 \cdot 7H_2O$, 2.94 g of $CaCl_2 \cdot 2H_2O$, 23.4 g of NaCl, 111 mg of $MnSO_4 \cdot 4H_2O$, 28.8 mg of $ZnSO_4 \cdot 7H_2O$, 29.2 mg of $Co(NO_3)_2 \cdot 6H_2O$, 25.2 mg of $CuSO_4 \cdot 5H_2O$, 24.2 mg of $Na_2MoO_4 \cdot 2H_2O$, 31.0 mg of H_3BO_3 and 4.53 g of trisodium EDTA per liter. The vitamin mixture was composed of 100 mg of nicotinic acid, 100 mg of thiamine hydrochloride, 5 mg of biotin, 50 mg of *p*-aminobenzoic acid, 1mg of vitamin B_{12}, 50 mg of calcium panthothenate, 50 mg of pyridoxine hydrochloride and 50 mg of folic acid per 100 ml. 1/5 PE medium is simply 5 times diluted PE medium. Ten times diluted PE medium (1/10 PE medium) or a hundred times diluted PE medium (1/100 PE medium) contained ten times or a hundred times lower concentration of sodium glutamate, sodium succinate, sodium acetate, yeast extract, casamino acids, sodium thiosulfate, $(NH_4)_2SO_4$, respectively. Both 1/10 and 1/100 PE medium contained the same concentrations of KH_2PO_4, K_2HPO_4, basal salt solution and 1/5 concentrations of vitamin mixture as the PE medium. Agar (1.5% w/v) or gellan gum (2% w/v) supplemented medium were designated to be PEA or PEG, respectively. Composition of Nutrient agar was 5.0 g of meat extract, 10.0 g of peptone, 5.0 g of sodium chloride and

15.0 g of agar per liter. The pH of the medium was adjusted to 7.0. Six times or 60 times diluted Nutrient agar (1/6 NA or 1/60 NA) were also used, supplied with agar up to 1.5%. Inoculated plates were cultivated aerobically at 30°C in the dark for 14 days.

After cultivation, randomly selected red or pink colored colonies were suspended in sterilized distilled water to determine absorption spectrum using spectrophotometer V-630 (Jasco, Tokyo, Japan). Colonies that showed absorbance maxima within 800 nm to 875 nm were selected and transferred to new agar plates of 1/10 PE medium and cultivated aerobically at 30°C in the dark for a week. Transfer and cultivation were repeated more than twice to obtain pure cultures. The winter isolates (February) were indicated by the ’W’ prefix, for example W09, and summer isolates (July), by the ‘S’ prefix.

Growth properties

Growth properties of isolates were determined under the following conditions. Anaerobic light conditions; Test tubes were sealed with butyl rubber caps and gas phase of each tube was substituted with nitrogen gas. Inoculated tubes were incubated under filtered incandescent light (ca. 2,000 lux) of wavelength over 700 nm. Aerobic dark conditions; Test tubes were equipped with silicon plug ‘SILICOSEN’ (Shin-etsu Polymer, Tokyo, Japan) to let air through, set in a shaker at an angle and shaken at 140 rpm in darkness. All cultivations were conducted 30°C, using test tubes of 18 mm in diameter and 180 mm in length containing 10 ml of liquid medium after inoculation of 0.1 ml of exponential phase of growth or early stationary phase in 1/5 PE medium under aerobic dark conditions. Growth was monitored by optical density of 660 nm (mini photo 518R, Taitec, Saitama, Japan). Five test tubes were used for each test.

Bacteriochlorophyll content

Isolates were cultivated under aerobic dark condition in 1/5 PE medium and the cells were harvested at exponential phase of growth or early stationary phase and washed twice with 10 mM tris-HCl buffer pH 8.0. Bacteriochlorophyll was extracted in methanol:acetone=7:2 from the cells and absorbance of 770 nm was measured using UV-1800 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). Millimolar extinction coefficient 75 cm^{-1} was used to determine bacteriochlorophyll content (3). Dry cell weight was determined with harvested cells, washed twice with 10 mM tris-HCl buffer pH 8.0 and dried 80^{o}C for 3 days.

Rhodobacter sphaeroides ATCC 17023^{T} (=2.4.1^{T}), a reference species obtained from the American Type Culture Collection was cultivated in 1/5 PE medium under aerobic dark and anaerobic light conditions.

Measurements of in vivo spectra

In vivo spectra of whole cells and disrupted cells were measured. For measurements of *in vivo* spectra of disrupted cells, 50 ml of an aerobic dark-grown 7 day cultures were used. Cultivations were conducted in vessels for shaken culture (500 ml capacity) containing 100ml of 1/5 PE medium. Cells were harvested and suspended in 10 mM Tris-HCl pH 8.0, disrupted by sonication at 130 W for 4 min in a chilled bowl (Sonicator, Ohtake Works, Tokyo, Japan) and centrifuged at 10,000 rpm for 4 min, the supernatant was used for *in vivo* absorption spectra measurements. Spectra were measured using UV-1800 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan).

Sequencing of the 16S rRNA gene and phylogenetic analysis

Total genomic DNA of the isolates was extracted according to Noll et al. (18). DNA fragments of partial 16S rRNA gene were amplified using primer sets 27F2/ 1492R2 (14, 23). DNA sequences were determined with the BigDye v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and a DNA sequencer ABI3130xl (Applied Biosystems). Sequence primers 27F2, 515F, 968F, 517R, 907R2 and 1492R2 were used and contig was made using ATGC program (GENETYX Ver. 12, GENETYX, Tokyo, Japan). Phylogenetic relatives were determined based on 16S rRNA sequences using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was conducted using MEGA version 6 program (24).

Results

Bacteriochlorophyll-containing colonies from epilithic biofilms

Table II-1 summarizes the results of isolation. 2.0 x 10^4 to 1.5 x 10^5 colonies per cm^2 of the biofilm were totally detected from the samples used in this study. The CFUs did not vary widely among media. Many red or pink colored colonies were obtained from both the February and July samples.

Among the colored colonies obtained from the sample in February, 17 colonies were picked up to determine bacteriochlorophyll. Thirteen colonies within 17 colonies had bacteriochlorophyll. For the sample collected in July, bacteriochlorophyll was detected from 15 colonies among 51 colonies tested in total. Using 1/10 PEA and 1/100 PEA media, bacteriochlorophyll-containing colonies were frequently obtained.

In vivo absorption spectra of whole cells of the 28 isolates showed absorption peaks within 800 nm to 875 nm. These peaks show the presence of a photosynthetic apparatus in which the bacteriochlorophyll is integrated. Fig. II-1 shows two characteristic *in vivo* absorption spectra of sonicated supernatant. The absorption spectrum of W09 showed absorption peaks at 803 nm and 871 nm, which indicate the presence of photochemical reaction center light-harvesting pigment-protein complex I (RC-LH I). W17 showed absorption peaks at 804 nm, 822 nm and 868 nm indicating the presence of RC-LH I and light-harvesting pigment-protein complex II (LH II) (4). Absorption peaks of 455 nm and 489 nm of W17 shows absorbance of carotenoid.

16S rRNA gene sequences of 28 bacteriochlorophyll-containing isolates were determined using the sequencing primer 27F2, and compared and classified the cut off

value of over 99.6% identity. 1409 positions of 16S rRNA gene sequences of representative 14 isolates (S08, W09, W32, W19, S19, W17, S16, S10, W45, S12, S20, W22, W14 and W35) were determined and finally these isolates were divided into 8 OTUs sharing 96% sequence identity. These OTUs were found in alpha-1, alpha-3, and alpha-4 subclasses of *Alphaproteobacteria* and *Betaproteobacteria* (details were shown in Fig. II-2).

Growth under anaerobic light conditions and aerobic dark conditions

Anaerobic growth was tested for representatives from phylogenetically distinct 8 OTUs and compared with that under aerobic conditions. Figure II-3 shows growth curves of three isolates, W35 (OTU8), S12 (OTU7) and W32 (OTU2) (Fig. II-3(A), (B) and (C)). All of these strains showed distinct growth under aerobic conditions in the dark (solid lines), and no growth under anaerobic conditions in the light (dashed lines). Other 5 isolates in OTUs 1, 3-6, also showed growth under aerobic conditions and no growth under anaerobic conditions in the light (Fig. II-S3A, II-S3B, II-S3C). All the representative isolates in this study were confirmed to be aerobic anoxygenic photosynthetic bacteria.

Phylogenetic analysis of the isolates

Fig. II-2 shows a neighbor-joining tree based on 16S rRNA gene sequences from 14 representative isolates obtained in this study together with those from the database. A phylogenetic tree using the maximum-likelihood method showed tree topology roughly consistent with that in Fig. II-2 (data not shown). 8 OTUs were distributed to alpha-1, alpha-3 and alpha-4 subclass of *Alphaporoteobacteria* and *Betaproteobacteria*.

Isolate S08 in OTU1 belonging to alpha-1 subclass was related to *Roseomonas lacus*, which had been isolated from freshwater sediment (10) with 98.1% identity.

Five isolates, W09, W32, W07, W40 and W19, i.e., OTU2 were found in alpha-3 subclass. *Tabrizicola aquatica* in this group had been isolated from a freshwater lake as a non-photosynthetic bacterium (25).

The alpha-4 subclass of *Proteobacteria*, OTUs 3-7, was the dominant group of the isolates. In the clade of OTUs 3-5, known aerobic anoxygenic photosynthetic bacteria such as *Sandarakinorhabdus limnophila* (5) was found.

OTU6 have neighbor *Sphingopyxis wooponensis*, isolated from freshwater of wetland.

Isolates belonging to OTU7 have aerobic anoxygenic photosynthetic relatives such as *Porphyrobacter donghaensis*.

An aerobic anoxygenic photosynthetic bacteria belonging to *Betaproteobacteria*, W35 (OTU8), was isolated. The nearest neighbor was aerobic anoxygenic photosynthetic bacteria *Aquincola tertiaricarbonis* isolated from German groundwater (16).

Nearest neighbors of the isolates and their similarities are listed in Table II-S1.

Growth properties in nutrient-rich medium and -poor medium

Growth affected by organic nutrients concentration in the medium was characterized. Three types of growth property were detected in 10 isolates (representatives of 8 OTUs and additional 2 isolates) under aerobic dark conditions (Fig. II-3). First type was approximately equal growth rate in PE medium and 1/5 PE medium as seen in W35 (Fig. II-3A), W09, W17, S16 and S10 (Fig. II-S3A). Second type

showed higher growth rate in PE medium compared to that in 1/5 PE medium as seen in S12 (Fig. II-3B), S08 and W45 (Fig. II-S3B). And third type indicated to be oligotrophic, i.e., grown in 1/5 PE medium but no growth in PE medium (isolates W32 and W19; Fig. II-3C and Fig. II-S3C). Differences in affinity to substrates may cause the difference in first type and second type.

Bacteriochlorophyll contents

Fig. II-4 shows bacteriochlorophyll contents of the isolates grown in 1/5 PE medium. The contents varied by strains from 0.13 to 3.58 nmol per mg of dry cell weight (Table II-S2). Within these, the highest content was comparable to that of anaerobic anoxygenic photosynthetic bacteria, *Rhodobacter sphaeroides* 2.4.1^{T}. Strains containing LHII (S19 and S16) (Table III-2, Fig. III-S1(g), (h) and (k)) had a tendency to have higher bacteriochlorophyll content.

Discussion

In this study, I isolated 28 strains of bacteriochlorophyll-containing bacteria from the river biofilms and characterized those strains as aerobic anoxygenic photosynthetic bacteria.

Phylogenetic analyses of 16S rRNA sequences of the isolates indicated that 8 OTUs were widely distributed in alpha-1, alpha-3 and alpha-4 subclasses of the class *Alphaproteobacteria* and the class *Betaproteobacteria* (Fig. II-1). These results suggest that a large diversity of aerobic anoxygenic photosynthetic bacteria is present in the river biofilm.

Among the 8 OTUs, four of them were supposed to be novel lineages (OTU1, OTU3, OTU6 and OTU8), as isolates belonging to these OTUs have 16S rRNA sequence similarities of 97.3 % to 98.1 % to known species.

Only a few lineages of aerobic anoxygenic photosynthetic bacteria have been known in *Betaproteobacteria*, and two genera, *Roseateles* (22) and *Aquincola* (19), have been described. The 16S rRNA sequence identity between the isolate W35 (OTU8) and the nearest neighbor *Aquincola tertiaricarbonis* (accession no. JX094174) was only 97.3%, and the isolate was obviously distinct from *A. tertiaricarbonis* in the phylogenetic tree (Fig. II-1). Therefore, the isolate W35 would represent a novel genus and species in *Betaproteobacteria*. Other than the isolate W35, several additional strains of anoxygenic photosynthetic bacteria in *Betaproteobacteria* have been isolated from the river biofilm taken from the sampling site 20 km upstream of that in this study (Chapter IV).

Five strains (W09, W32, W07, W40 and W19) belonging to the alpha-3 subclass

of *Alphaproteobacteria* (OTU2) were isolated from the biofilm collected in winter (Fig. II-1). This OTU of the isolates form a different clade from any known aerobic anoxygenic photosynthetic bacteria in the alpha-3 subclass such as *Roseobacter denitrificans* and *Roseisalinus antarctis.* The isolates form a clade with *Rhodobacter* species able to grow photosynthetically under anaerobic conditions. Until now, all aerobic anoxygenic photosynthetic bacteria belonging to the alpha-3 subclass of *Alphaproteobacteria* are marine or halophilic organisms classified *Roseobacter* clade (28, 29). Our new isolates in the alpha-3 subclass were the first freshwater organisms and also the first aerobic anoxygenic photostrophic bacteria belonged to the clade other than the *Roseobacter* clade.

The two isolates W19 and W32, belonging to the OTU2, showed significant growth in the low nutrient medium but no growth in the high nutrient medium (Fig. II-2C, Fig. II-S2C). These strains may have adapted to the oligotrophic environments and lost the ability to grow in high-nutrient conditions. On the other hand, the rest of the isolates grew well in the high nutrient PE medium. The bacteriochlorophyll-content of isolate S10 was only 0.13 nmol/mg, whereas that of S16 was 3.58 nmol/mg. Bacteriochlorophyll contents varied greatly depending on strains (Fig. II-3, Table II-S3). These physiological diversities in nutrient requirement and bacteriochlorophyll contents may indicate that there are various microniches for aerobic anoxygenic photosynthetic bacteria in the river biofilm, i.e., diverse microenvironments such as different light conditions and concentrations of organic and inorganic chemicals depending on the space and time.

In conclusion, the present work showed the presence of phylogenetically and physiologically diverse aerobic anoxygneic photosynthetic bacteria in the river biofilm.

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purple phototrophic bacteria. Springer Science, Dordrecht, Netherlands.

Table II-1. Numbers of total colonies, colored colonies and BChl-containing colonies

Media	February 2012			July 2012		
	Total	Red or pink colored		Total	Red or pink colored	
	CFU	CFU (%)*	BChl**	CFU	CFU (%)*	BChl**
PEA				7.6×10^4	6.0×10^4 (79)	2/10
1/10PEA	1.5×10^5	1.2×10^5 (79)	8/8	8.4×10^4	3.4×10^4 (40)	6/12
1/10PEG	1.0×10^5	2.0×10^4 (20)	1/2	2.0×10^4	2.9×10^3 (15)	0/2
1/100PEA	3.1×10^4	6.7×10^3 (21)	2/2	6.2×10^4	2.1×10^4 (35)	4/5
1/100PEG	2.0×10^4	6.7×10^3 (33)	2/5	4.0×10^4	1.5×10^4 (36)	2/8
NA				3.3×10^4	2.9×10^4 (89)	0/1
1/6NA				4.4×10^4	2.7×10^4 (61)	0/7
1/60NA				6.1×10^4	2.4×10^4 (39)	1/6

CFU : Colony forming unit (/cm^2 biofilm)

PEA, PEG : PE medium supplemented with agar (PEA) or gellan gum (PEG).

NA : Nutrient agar (containing 5g of meat extract,10g of peptone and 5g of NaCl per liter)

(%)* : Percentage of colored CFU/total CFU

BChl** : Bacteriochrolophyll-containing colonies/colored colonies tested

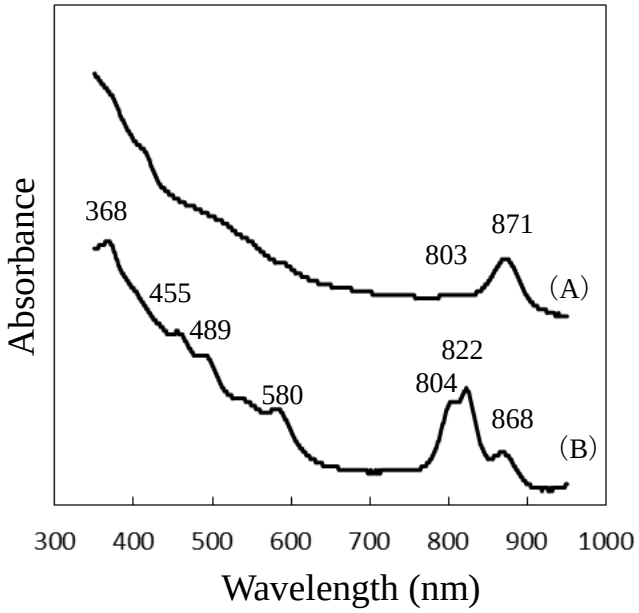

Fig. II-1. *In vivo* absorption spectra of strains W09 (A) and W17 (B).
Cells were cultivated aerobically in the dark for 7 days using 1/5 PE medium. Harvested cells were washed and sonicated in 10mM Tris-HCl buffer pH8.0. The centrifuged supernatant were used to measure absorption spectra. The absorption spectra were measured using UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan).

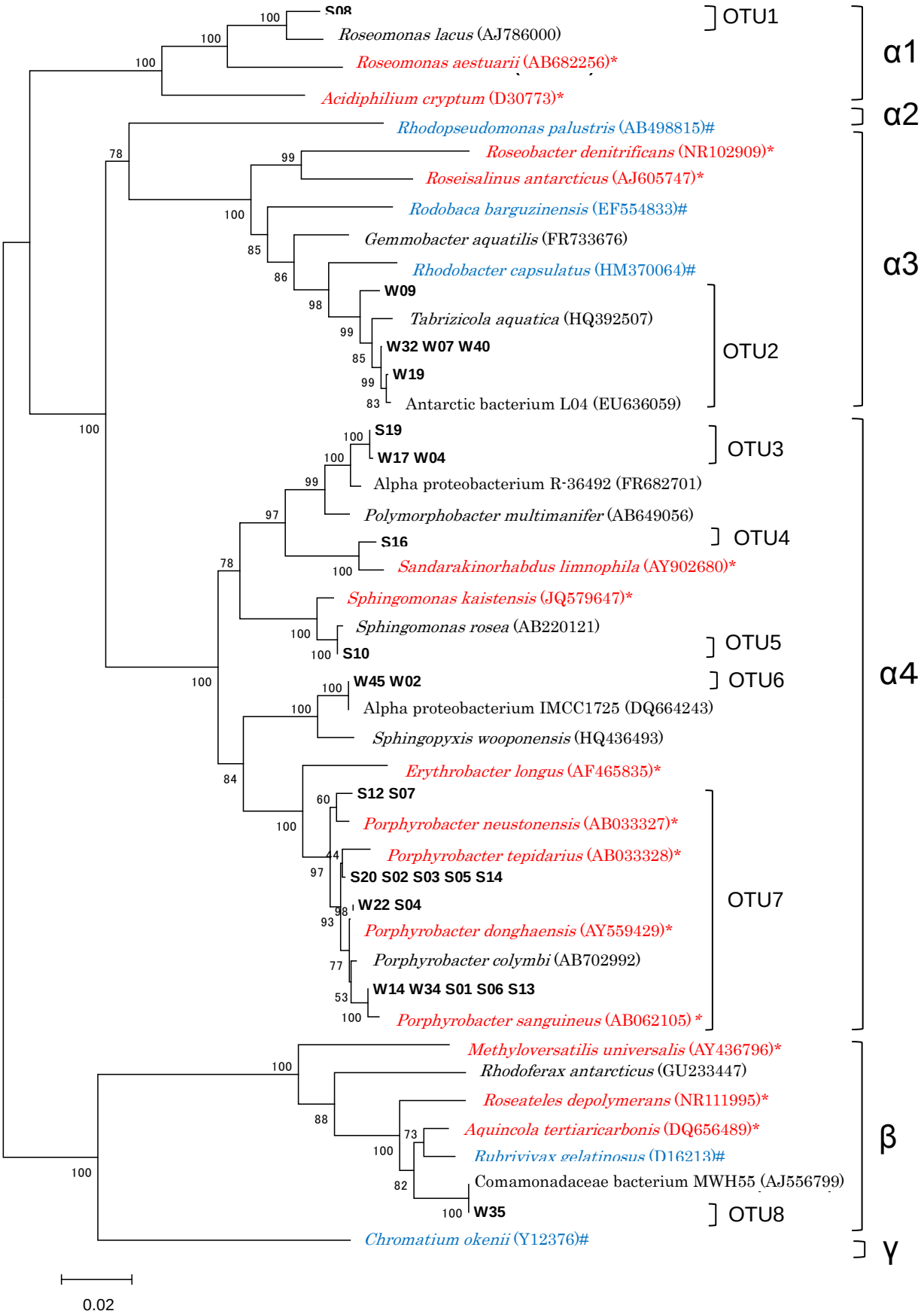
S08
Roseomonas lacus (AJ786000)
Roseomonas aestuarii (AB682256)*
Acidiphilium cryptum (D30773)*
Rhodopseudomonas palustris (AB498815)#
Roseobacter denitrificans (NR102909)*
Roseisalinus antarcticus (AJ605747)*
Rodobaca barguzinensis (EF554833)#
Gemmobacter aquatilis (FR733676)
Rhodobacter capsulatus (HM370064)#
W09
Tabrizicola aquatica (HQ392507)
W32 W07 W40
W19
Antarctic bacterium L04 (EU636059)
S19
W17 W04
Alpha proteobacterium R-36492 (FR682701)
Polymorphobacter multimanifer (AB649056)
S16
Sandarakinorhabdus limnophila (AY902680)*
Sphingomonas kaistensis (JQ579647)*
Sphingomonas rosea (AB220121)
S10
W45 W02
Alpha proteobacterium IMCC1725 (DQ664243)
Sphingopyxis wooponensis (HQ436493)
Erythrobacter longus (AF465835)*
S12 S07
Porphyrobacter neustonensis (AB033327)*
Porphyrobacter tepidarius (AB033328)*
S20 S02 S03 S05 S14
W22 S04
Porphyrobacter donghaensis (AY559429)*
Porphyrobacter colymbi (AB702992)
W14 W34 S01 S06 S13
Porphyrobacter sanguineus (AB062105) *
Methyloversatilis universalis (AY436796)*
Rhodoferax antarcticus (GU233447)
Roseateles depolymerans (NR111995)*
Aquincola tertiaricarbonis (DQ656489)*
Rubrivivax gelatinosus (D16213)#
Comamonadaceae bacterium MWH55 (AJ556799)
W35
Chromatium okenii (Y12376)#
OTU1
OTU2
OTU3
OTU4
OTU5
OTU6
OTU7
OTU8
α1
α2
α3
α4
β
γ
0.02

Fig. II-2.

Fig. II-2. Phylogenetic tree based on 16S rRNA sequences. 16S rRNA sequences of strains isolated in this study are indicated in bold by the “W” and “S” prefix in the case of winter and summer isolates, respectively. Sequences from the database are represented with their respective accession numbers after bacterial name in parentheses. Within these, the bacteria which were known to be aerobic anoxygneic photosynthetic bacteria were marked with asterisk (*) beside the accession numbers and indicate by red type. The bacteria which were known to be anaerobic anoxygenic photosynthetic bacteria were marked with sharp (#) beside the accession numbers and indicate by blue type. OTUs are indicated to the right of the tree. Alpha-1, alpha-2, alpha-3 and alpha-4 subclass, beta and gamma class of *Proteobacteria* are also indicated by α1, α2, α3, α4, β and γ, respectively to the right of the tree. The scale bar represents number of substitutions per site.

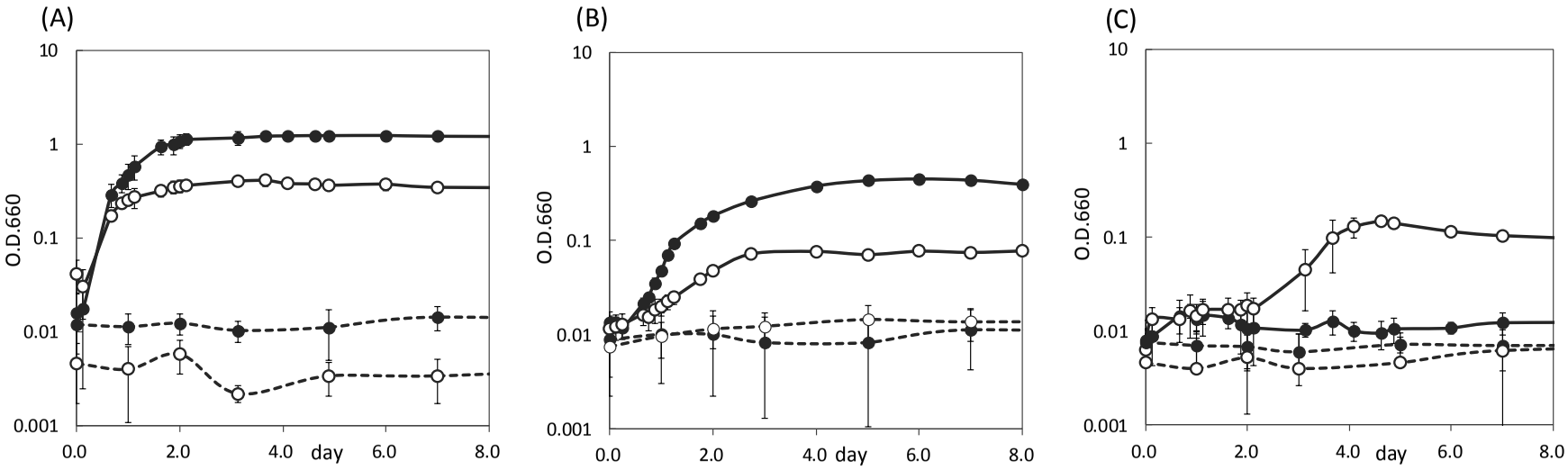

Fig. II-3. Growth curves of the isolate W35 (A), S12 (B) and W32 (C). Cells were cultivated aerobically in the dark (continuous line) and anaerobically in the light (dashed line) using PE medium (closed circle) and 1/5 PE medium (open circle). Error bar shows the standard deviation.

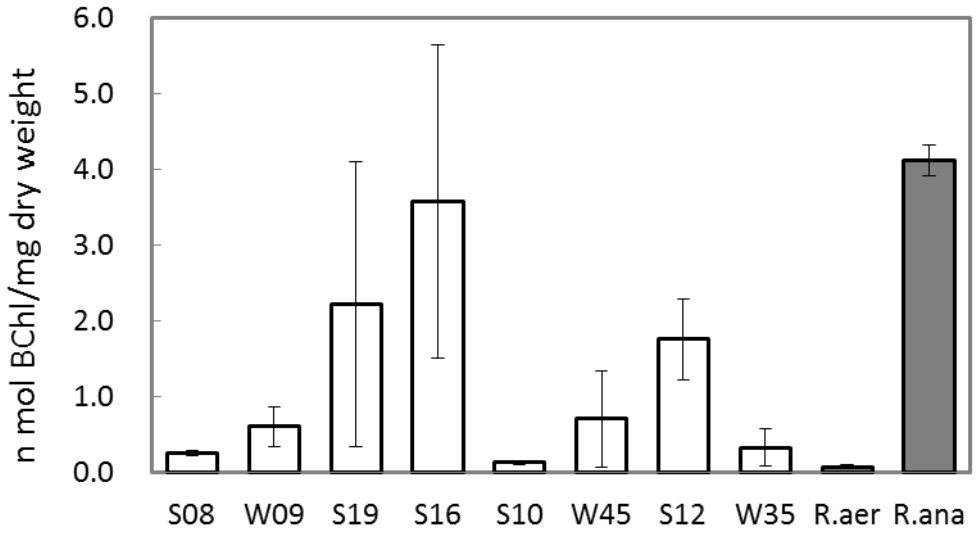

Fig. II-4. Bacteriochlorophyll contents of the isolates and anaerobic purple photosynthetic bacterium *Rhodobacter sphaeroides*

All cultivations were conducted using 1/5 PE medium.

Each isolates were cultivated aerobically in the dark.

R.aer : *Rhodobacter sphaeroides* cultivated aerobically in the dark.

R.ana : *Rhodobacter sphaeroides* cultivated anaerobically in the light.

Cultivation and measurements were conducted three times for each strain and the averages were shown. Error bar shows the standard deviation of three times of cultivation.

Table II-S1. Phylogenetic relatedness of the isolates to described species

Strain	Isolation medium	Affiliation	Nearest neighbor			Other strains (isolation medium number)	Colony color
			Accession no.		Similarity (%)		
S08	1	α1(OTU1)	AJ786000	Roseomonas lacus	1349/1375 (98.1%)	-	pink-orange
W09	3	α3(OTU2)	HQ392507	Tabrizicola aquatica	1366/1388 (98.4%)	-	pink beige
W19	1	α3(OTU2)	HQ392507	Tabrizicola aquatica	1373/1390 (98.8%)	-	pink beige
W32	3	α3(OTU2)	HQ392507	Tabrizicola aquatica	1377/1388 (99.2%)	W07(1) W40(1)	pink beige
S19	1	α4 (OTU3)	AB649056	Polymorphobacter multimanifer	1390/1418 (98.0%)	-	brown
W17	1	α4 (OTU3)	AB649056	Polymorphobacter multimanifer	1387/1416 (98.0%)	W04(1)	yellow-brown
S16	3	α4 (OTU4)	AY902680	Sandarakinorhabdus limnophila*	1327/1346 (98.6%)	-	red-brown
S10	6	α4 (OTU5)	AB220121	Sphingomonas rosea	1420/1422 (99.9%)	-	pink-orange
W45	1	α4 (OTU6)	HQ436493	Sphingopyxis wooponensis	1398/1430 (97.8%)	W02(1)	yellow-orange
S12	5	α4 (OTU7)	AB033327	Porphyrobacter neustonensis*	1424/1436 (99.2%)	S07(3)	brown-red
S20	5	α4 (OTU7)	KC329833	Porphyrobacter neustonensis*	1427/1438 (99.2%)	S02(1) S03(4) S05(3) S14(1)	pale red-brown
W22	2	α4 (OTU7)	AY559429	Porphyrobacter donghaensis*	1420/1420 (100%)	S04(4)	orange-brown
W14	1	α4 (OTU7)	AB702992	Porphyrobacter colymbi	1420/1428 (99.4%)	W34(4) S01(1) S06(3) S13(1)	brown-red
W35	4	β(OTU8)	DQ656489	Aquincola tertiaricarbonis*	1412/1451 (97.3%)	-	beige

Nearest neighbor* indicates aerobic photosynthetic bacteria.

Isolation medium 1:1/10PEA 2:1/10PEG 3:1/100PEA 4:1/100PEG 5:PEA 6:1/60NA

"Other strains" are strains having over 99.6% sequence similarity to representative strain.

Table II-S2. Bacteriochlorophyll contents of the isolates and purple photosynthetic bacterium *Rhodobacter sphaeroides*

		Bacteriochlorophyll/Dry weight（nmol/mg）			
		PE medium		1/5PE medium	
Affiliation	Strain name	BChl/DW	STDEVA	BChl/DW	STDEVA
α1	S08	0.44	0.05	0.26	0.04
α3	W09	0.29	0.18	0.61	0.26
α4（OTU3)	S19	0.95	0.21	2.23	1.88
α4（OTU4)	S16	2.90	1.22	3.58	2.07
α4（OTU5)	S10	0.00	0.00	0.13	0.02
α4（OTU6)	W45	0.15	0.09	0.71	0.64
α4（OTU7)	S12	1.53	0.13	1.76	0.53
β	W35	0.07	0.03	0.33	0.25
	R.sph aerobic	0.04	0.03	0.08	0.03
	R.sph anaerobic	10.30	0.10	4.12	0.21

Each isolates were cultivated aerobically in the dark in liquid medium PE or 1/5 PE.
Cells were harvested in exponential phase of growth or early stationary phase.
R.sph aerobic: *Rhodobacter sphaeroides* cultivated aerobically in the dark.
R.sph anaerobic: *Rhodobacter sphaeroides* cultivated anaerobically in the light.
BChl/DW: average value of three measurements.
STDVA: standard deviation

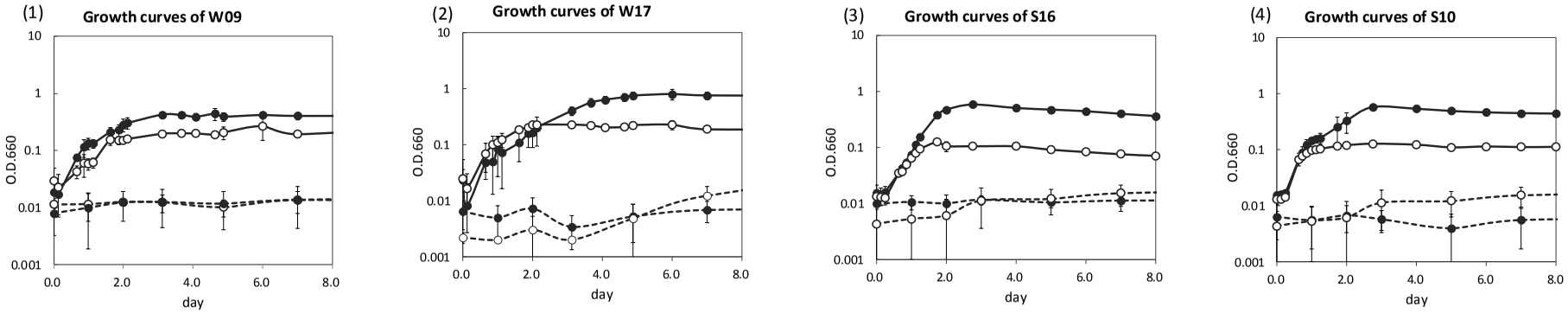

Fig. II-S3A. Growth curves of the isolate W09 (1), W17 (2), S16 (3) and S10 (4). Cells were cultivated aerobically in the dark (continuous line) and anaerobically in the light (dashed line) using PE medium (closed circle) and 1/5 PE medium (open circle). Error bar shows the standard deviation

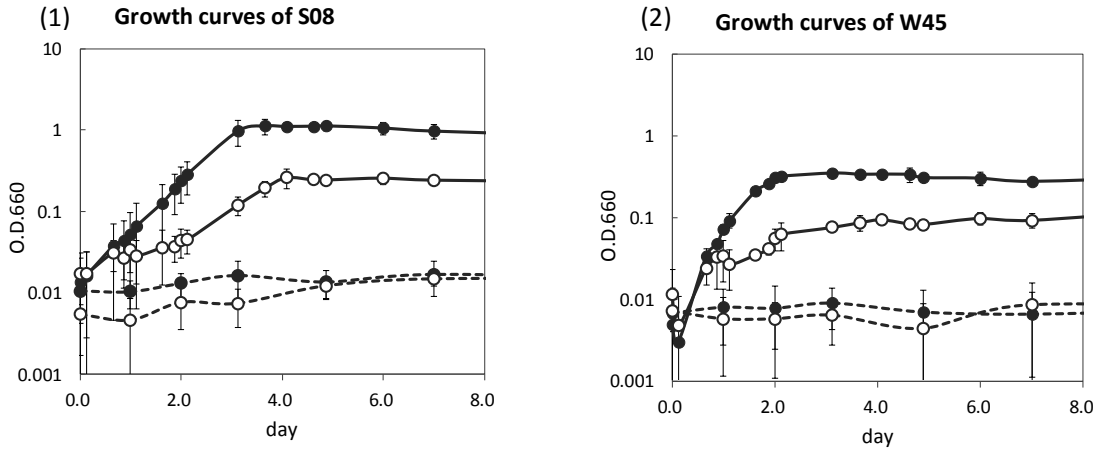

Fig. II-S3B. Growth curves of the isolate S08 (1) and W45 (2). Cells were cultivated aerobically in the dark (continuous line) and anaerobically in the light (dashed line) using PE medium (closed circle) and 1/5 PE medium (open circle). Error bar shows the standard deviation.

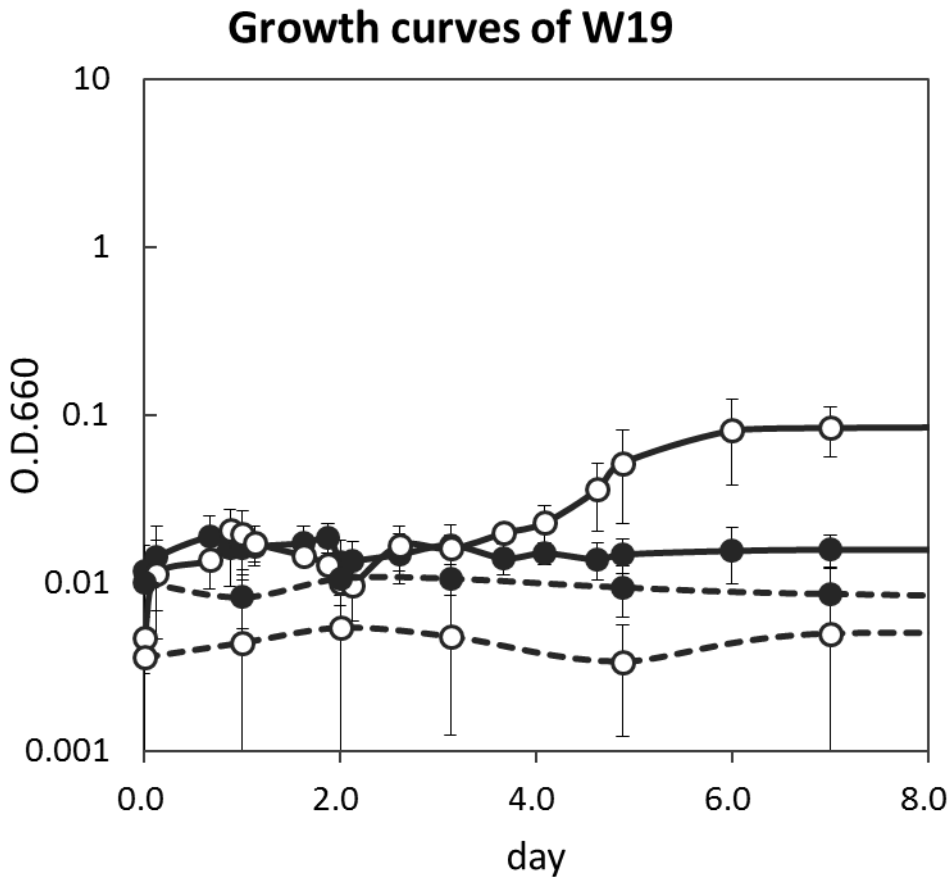

Fig. II-S3C. Growth curves of the isolate W19. Cells were cultivated aerobically in the dark (continuous line) and anaerobically in the light (dashed line) using PE medium (closed circle) and 1/5 PE medium (open circle). Error bar shows the standard deviation.

CHAPTER III

Physiological Properties of Novel Aerobic Anoxygenic Photosynthetic Bacteria Isolated from Epilithic Biofilms in Tama River in Ohme-city, Japan

Abstract

Physiological, biochemical and morphological properties of aerobic anoxygenic photosynthetic bacteria isolated from Tama River described in Chapter II were characterized.

Strain S08 phylogenetically belongs to the genus *Roseomonas* in alpha-1 subclass of *Alphaproteobacteria*. S08 produced pink-orange pigment and *Roseomonas* species produce pigments of similar color. So far only one photosynthetic species *Roseomonas aestualii* has been described in the genus, and strain S08 has only 93.4% similarity to the species. S08 grew at 10-35^{O}C, but in the case of *R. aestualii* and the nearest neighbor of S08, *Roseomonas lacus*, growth occured even at 40^{O}C.

Strains W09, W19 and W32 belong to the alpha-3 subclass of *Alphaproteobacteria* and have the nearest neighbor *Tabrizicola aquatica* with 99.2% identity. But the neighbor was reported to have neither bacteriochlorophyll nor *puf* genes. The isolates and *T. aquatica* also differed in the growth temperature. The isolates grew at 10^{O}C to 35^{O}C, with the optimum growth at 25^{O}C to 30^{O}C, but *T. aquatica* was reported to grow at 15^{O}C to 55^{O}C, with the optimum growth at 40^{O}C to 45^{O}C.

Strains S19, W17 and S16 belong to the alpha-4 subclass of *Alphaproteobacteria*. Spectroscopic analyses revealed that these strains had light harvesting pigment protein complex II (LH II) together with LH I like their phylogenetic relative *Sandarakinorhabdus limnophila*. Strains S19 and W17 grew at low temperature 10^{O}C to 30 ^{O} C but did not grow at 35 ^{O} C. These isolates and their closest relative *Polymorphobacter multimanifer* isolated from Antarctica differed in the possession of

bacteriochlorophyll and in morphology. Strain S16 grew at 10-35^{O}C. The isolate differs from the nearest relative *S. limnophila* in the cell size, colony color and the wavelength of absorption peak of LH II.

Strain W35 belongs to the class *Betaproteobacteria* and differed from the closest relative, an aerobic anoxygenic photosynthetic bacterium *Aquincola tertiaricarbonis,* in their cell size, growth temperature and colony color.

Introduction

Aerobic anoxygenic photosynthetic bacteria were first described in 1978 (4, 18) as obligate aerobic marine bacteria containing bacteriochlorophyll, and then have been isolated from a variety of environments. The majority of those bacteria have wide distribution in the phylogenetic lineage of the class *Alphaproteobacteria*. The members were also found in the classes *Betaproteobacteria* (12, 17, 20) and *Gammaproteobacteria* (23) and as well as in the phylum *Gemmatimonadetes* (24). They have been reported to be widespread in the ocean and have significant roles in the carbon cycling and energy supply from sunlight to the marine ecosystem (8, 9). But less is known about aerobic anoxygneic photosynthetic bacteria in the freshwater environments.

I had isolated 28 strains of aerobic anoxygenic photosynthetic bacteria from river biofilms including many possibly novel species (Chapter II). Physiological properties of the new isolates obtained in the study described in Chapter II should be usefull for the understandings of aerobic anoxygenic photosynthetic bacteria in rivers, as only one species of aerobic anoxygenic photosynthetic bacteria have been described from freshwater rivers (20).

In this study, for the further description of novel isolates obtained in the study described in Chapter II, their cell size and shape, colors of colonies, temperature range of growth and thermotolerance were examined. To know what types of pigment-protein complexes the isolates have, absorption peaks of *in vivo* absorption spectra were also measured. In addition, *pufM* gene sequences coding for a subunit of the photochemical reaction center of the isolates were determined.

The isolated 28 strains were phylogenetically divided into 8 OTUs, of which 4 OTUs were distantly related to the known species. One OTU other than these 4OTUs, belonging to the alpha-3 subclass of *Alphaproteobacteria* has the closest relative, which was reported to have no bacteriochlorophyll.

Materials and Methods

Bacterial strains

All the bacterial strains used in this chapter were the aerobic anoxygenic photosynthetic bacteria isolated from Tama River, Japan described in Chapter II. Strains used were as follows, strain S08 belonging to the alpha-1 subclass of *Alphaproteobacteria*, strains W09, W19 and W32 belonging to the alph-3 subclass of *Alphaproteobacteria*, strains S19, W17 and S16 belonging to the alph-4 subclass of *Alphaproteobacteria*, W35 belonging to the class *Betaproteobacteria*. For *puf* gene amplification, all the strains isolated in Chapter II were used.

Observations of cell shape and measurements of cell size

Cells of aerobically grown colonies on 1/10 PE agar medium in the dark were suspended in sterilized phosphate buffer and observed under a microscope (Imager.A2, Carl Zeiss, Oberkochen, Germany). Length and width of 10 cells were measured and the range was shown for each strain.

Measurements of the range of growth temperature and tolerance to low and high temperatures

Bacterial cells were cultured in 1/5 PE medium aerobically in the dark at 30^{O}C. Each culture of the exponential phase of growth or the early stationary phase was inoculated to two agar plates of the PE medium and the 1/10 PE medium (the compositions were described in Chapter II). The inoculated plates were incubated at 0^{O} C, 4^{O}C, 10^{O}C, 15^{O}C, 20^{O}C, 25^{O}C, 30^{O}C, 35^{O}C, 40^{O}C, 45^{O}C and 50^{O}C. After 14 days,

growth was checked for the formation of colonies.

The plates on which colonies were not detected after 14 days were transferred to an incubator at 30^{O}C, and growth was checked again by colony formation after another 14 days incubation.

In vivo *absorption peaks of the isolates*

For the measurements of *in vivo* absorption spectra, bacterial cells were cultivated, harvested, disrupted and absorption spectra were measured as described in Chapter II.

Genomic DNA extraction, amplification of puf *genes and phylogenetic analysis*

Bacterial strains were cultured aerobically in the dark at 30^{O}C in PE medium or 1/5 PE medium. Total genomic DNAs were extracted from harvested cells, according to Noll *et al.* (14). DNA fragments of *pufLM* genes coding for the L and M subunits of the photochemical reaction center were amplified. Polymerase chain reaction (PCR) was conducted to amplify *pufLM* gene fragments from DNA of isolated cells using the primer sets of pufLM-F/pufLM-R (13). The reaction mixture contained 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 0.5 μM each primer, 0.2 to 1 μL of temperate DNA, 1 x Ex Taq buffer and 0.5 u of Ex Taq (TaKaRa, Shiga, Japan) in a total volume of 20μL. PCR was performed in a 2720 Thermal Cycler (Applied Biosystems, Foster city, CA, USA). PCR conditions were as follows: initial denaturation for 3 min at 94.0^{O}C, and 30 cycles of 0.5 min of denaturation at 94.0^{O}C, 1 min of annealing at 55^{O}C and 1.5 min of extension at 72^{O}C, and post-run of extension at 72^{O}C for 3.3 min. Lower annealing temperatures, 53^{O}C, 50^{O}C and 48^{O}C were also

tested. After PCR, products were applied to agarose gel electrophoresis to confirm if the expected sizes of DNA fragments were amplified. DNA sequences were determined with the BigDye v3.1 cycle sequencing kit (Applied Biosystems, Foster city, CA, USA) and a DNA sequencer ABI3130xl (Applied Biosystems).

The phylogenetic tree based on the amino acid sequences of the partial PufM was constructed using the neighbor-joining and maximum-likelihood methods with a MEGA version 6 program (21).

Results

Cell shape and size

As shown in Table III-1, cells of all strains were short rod shaped, with 0.5-1.1 μm in width and 0.8-1.5 μm in length. While isolate W19 was observed as a single cell or often formed in chain with 2 to 4 cells, all other strains were observed as single cells. All strains did not show any motility under the conditions.

In vivo absorption peaks

In vivo absorption spectrum of each strain showed an absorption peak or a shoulder around 860-870 nm (Table III-2, Fig. III-S1). Wavelengths of absorption peaks differ among strains. Some strains (S19, W17, S16) have 2 peaks or a peak with a shoulder around 865 nm other than a peak around 800 nm (Fig. III-S1), showing 2 types of the light-harvesting pigment protein complexes LH I and LH II. Strain W19 have relatively high 804 nm peak, showing to have Roseobacter-type LH II together with LH I (19). The others (S08, W09, W32, W35) have only one type of the pigment protein complex LH I.

Spectroscopic analysis of *in vivo* absorption spectra showed all of the isolates have carotenoids in addition to bacteriochlorophyll (Table III-2, Fig. III-S1).

Colors of colonies of some strains isolated in the study described in Chapter II were listed in Table II-S1. As are the previously reported aerobic anoxygenic photosynthetic bacteria, colonies of the isolates showed pale colors such as pink-orange and pink-beige.

Growth temperature

As described in Materials and Methods, growth was observed by colony formation on agar plates. Table III-3 summarized the growth of the strains at various temperatures. Most strains grew in the range of 10^{O}C to 35^{O}C. All the strains tested did not show growth at 4^{O}C after 14 days, but after the plates were transferred to 30^{O}C, they grew shortly. All the tested strains also did not grow at 40^{O}C or higher temperatures, and they did not grow after transferring to 30^{O}C. Furthermore, two strains, S19 and W17, did not grow at 35^{O}C for 14 days and after transferring to 30^{O}C they did not grow neither (Table III-3, details are listed in Table III-S1).

Amplification of pufLM *genes*

All the isolated strains obtained from the study in Chapter II were analyzed for *pufLM* genes by PCR amplification. Although spectroscopic evidences showed all the isolates have bacteriochlorophyll which integrated in proteins, the majority of *pufLM* genes in these bacteria was not successfully amplified. However, the attempt was succeeded in four strains belonging to OTU6 (W45) and OTU7 (S05, S12 and S20). All the amplified isolates belong to alpha-4 subclass of *Alphaproteobacteria*. The phylogenetic analysis of *pufLM* genes showed the closest relative of strain W45 was clone TP-42 obtained from the study in Chapter I. The closest relative of S12 was *Porphyrobacter neustonensis* (accession no. AB011073) and that of S05 and S20 was *Porphyrobacter tepidarius* (accession no. AB020599) (Fig. III-S2). These four strains were placed in the clade of alpha-4 subclass in the phylogenetic tree based on *puf*

sequences (Fig. III-S2). These results were consistent with those of the phylogenetic analyses of these bacteria based on 16S rRNA gene sequences (Fig. II-1). No isolate did not show high similarity to the clone obtained in Chapter I.

Discussion

Morphologically all the studied strains were rod shaped with 0.5 to 1.1 μm in width and 0.8 to 1.5 μm in length. Spectroscopic analyses showed some strains have light-harvesting pigment protein complex II (LH II) together with LH I, whereas others have only LH I.

High-temperature sensitivity was observed in all the isolates examined. From the literature, the temperature ranges of growth and the optimum growth temperature of several aerobic anoxygenic photosynthetic bacteria were listed in Table III-4 together with their habitats. Upper limit of the growth temperature in the species listed in Table III-4 is 37^{O}C to 43.5^{O}C except for *Staleya gattiformis* isolated from Antarctica. Two strains of the present isolates did not grow at 35^{O}C. Other isolates grew at 35^{O}C but did not grow at 40^{O}C. These upper limits are lower than those of most aerobic anoxygenic photosynthetic bacteria listed in Table III-3 except that from Antarctica. Temperature of the river water in the upper region of Tama river is kept low throughout the year (4.6^{O} C-20.2 ^{O} C at Wadabashi, 2.5km upstream of the sampling site in 2012) (http://www.kankyo.metro.tokyo.jp/). The low temperature in river water of the sampling site may affect the high temperature sensitivity in the growth of the isolates.

Isolate S08 belonging to the alpha-1 subclass of *Alphaproteobacteria*, has relatively low (98.1%) identity to the nearest neighbor *Roseomonas lacus* (accession no. AJ786000), and has low identity 93.4% to the nearest aerobic anoxygenic photosynthetic bacteria *Roseomonas aestuarii* (accession no. AB682256) (Chapter II). *R. aestualii* is the only aerobic anoxygenic bacteria reported in the genus. Properties of isolate S08, *R. lacus* and *R. aestualii* were compared in Table III-5. Isolate S08 had

absorption peaks at 798 nm and 866 nm, corresponding to LH I whereas *R. lacus* was not reported to have bacteriochlorophyll. Isolate S08 and *R. lacus* differ in their growth temperature and the colony colors although the cell size is almost the same. The temperature range of growth of S08 was between 10^{O}C to 35^{O}C whereas that of *R. lacus* was between 15^{O}C to 40^{O}C. The colony color of S08 was pink-orange whereas that of *R. lacus* was colorless to pale-pink (7). Also the properties of S08 differ from those of the nearest aerobic anoxygenic photosynthetic bacteria *R. aestualii* in growth temperature and cell size. The temperature range of growth of *R. aestualii* was from 20^{O}C to 40^{O}C. S08 showed short rod shape with 0.7-0.8 μm in width and 1.1-1.5 μm in length whereas *R. aestualii* has a longer cell, 0.6-1.0 μm in width and 1.5-2.0 μm in length, *in vivo* absorption maxima of *R. aestualii* was not reported (16).

Isolates W09, W19 and W32 belong to alpha-3 subclass of *Alphaproteobacteria* and have the nearest neighbor *Tabrizicola aquatica* (accession no. HQ392507) with 99.2% identity, but the neighbor was reported to have neither bacteriochlorophyll nor *puf* genes. These isolates are the first freshwater aerobic anoxygenic photosynthetic bacteria in alpha-3 subclass of *Alphaproteobacteria* and also the first aerobic anoxygenic photosynthetic bacteria belonged to the clade other than the *Roseobacter*-clade. Also 2 strains of them, W19 and W32, showed growth properties of oligotrophs (Chapter II). Table III-6 showed the comparison of W09, W19 and W32 with *T. aquatica*. W09, W19 and W32 had absorption peaks around 800 nm and at 871 nm (W09), at 804 nm and 866 nm (W19), and at 802 nm and around 865 nm (W32). Absorption spectra of W09 and W32 showed that the strains have only one type of light harvesting pigment protein complex LH I, whereas W19 have high peak at 804 nm

showing this strain have Roseobacter-type LH II (19) other than LH I (Fig. III-S1). One of the big differences between the isolates and the nearest relative *T. aquatica* was the difference in growth temperature. Temperature range for growth of the isolates was from 10^{O}C to 35^{O}C with the optimum temperature of 25^{O}C to 30^{O}C, whereas that of *T. aquatica* was from 15^{O}C to 55^{O}C with the optimum temperature of 40^{O}C to 45^{O}C. The species has reported to have no bacteriochlorophyll, larger cells, 0.9 μm in width and 1.3-3 μm in length (the isolates have cells of 0.5-0.7 μm in width and 0.9-1.2 μm in length), showed colony color of colorless or cream (22) (the isolates showed colony color of pink beige). As similarity in 16S rRNA sequences between them is 99.2%, the isolates and *T. aquatica* are likely to be the same species, the large differences in their properties like growth temperature are anomalous. Careful comparison between the reported species and the new isolates may be useful for further clarification.

Isolates S19 and W17, which belong to the alpha-4 subclass of *Alphaproteobacteria*, have 98.0% identity to the nearest neighbor, *Polymorphobacter multimanifer* (accession no. AB649056), and have only 95.2% identity to the nearest photosynthetic neighbor, *Sandaracinorhabdus limnophila* (accession no. AY902680) (Chapter II). Table III-7 showed the comparison of S19 and W17 with *P. multimanifer* and *S. limnophila*. The temperature range of growth of the isolates was low, 10^{O}C to 30 ^{O}C, similar to that of the neighbor, *P. multimanifer*. *P. multimanifer* was isolated from a crack of white rock collected in Antarctica and grew at temperatures between -4 and 30^{O} C. The isolates and *P. multimanifer* differ in the possession of bacteriochlorophyll and morphology. The isolates were short-rod shaped, whereas the species showed morphological diversity in response to growth conditions, for example, circular or ovoid

cells in the lag phase and short-and long-rod shaped morphology on agar plate medium. S19 and W17 had absorption peaks at 801 nm, around 838 nm and 867 nm (S19), at 804 nm, around 822 nm and 868 nm (W17), indicating the presence of LH I and LH II. Although identity in the sequences of 16S rRNA gene between S19 and W17 was 99.93%, the wavelengths of an absorption peak of LH II (838 nm and 822 nm) were different from each other (Table III-7). Although 16S rRNA sequence similarity is as low as 95.2%, the wavelength of the peaks of the absorption spectrum of the isolates resembled to the nearest photosynthetic relative, *Sandarakinorhabdus limnophila*, but the cell size and the colony color differed. *S. limnophila* is aerobic anoxygneic photosynthetic bacteria and shows *in vivo* absorption maxima at 800, 837 and 865 nm, having LH I and LH II. The isolates showed a short rod shape with 0.5-0.6 μm in width and 1.0-1.5 μm in length, whereas reported strain has a smaller cell, 0.25-0.47μm in width and 0.61-0.99 μm in length. Colony color of the isolates was brown whereas that of the species was orange-red. The temperature range of growth of the species was not described (3).

Isolate S16, which belongs to the alpha-4 subclass of *Alphaproteobacteria*, have 98.6% identity to the nearest neighbor, *S. limnophila* (accession no. AY902680). The isolate have a large amount of bacteriochlorophyll, which is comparable to the anaerobic photosynthetic bacteria (Chapter II). S16 is phylogenetically related to S19 and W17. The properties differ from the nearest relative *S. limnophila* in the cell size, colony color and the wavelength of absorption peak of LH II and differ from S19 and W17 in the colony color and the wavelength of absorption peak of LH II (Table III-7). S16 showed cell size of 0.5-0.7 μm in width and 1.2-1.5 μm in length, a little bigger than *S. limnophila*, and about the same to S19 and W17. The wavelength of absorption

peak of LH II of 853 nm was longer than *S.limonophila*, S19 and W17. The colony color was red-brown.

Isolate W35 belongs to *Betaproteobacteria*, in which only a few aerobic anoxygenic photosynthetic bacteria have been reported (12, 17, 20). The properties of W35 were compared to the nearest relative *Aquincola tertiaricarbonis* (Table III-8). W35 differs from the aerobic anoxygneic photosynthetic bacterium *A. tertiaricarbonis* in cell size, growth temperature, colony color and conditions of pigment production. W35 show a short rod shape with 0.8-1.1 μm in width and 0.8-1.5 μm in length, have an absorption peak at 871 nm, the temperature range of growth from 10^{O}C to 35^{O}C, and colony color of beige. *Aquincola tertiaricarbonis* has relatively longer cell, 0.8-1 μm in width and 1.2-2 μm in length, shows the temperature range of growth from 4^{O}C to 40^{O}C, and the colony color of white. This species synthesize bacteriochlorophyll only under the extremely low nutrient conditions, and the in vivo absorption peak of this species was not described (12, 17), whereas W35 produce bacteriochlorophyll at all times, even when grown in PE medium, which contain as high as 2.5 g L^{-1} of organic compounds.

As described above, many isolates described in Chapter II are supposed to be distinct not only phylogenetically but also physiologically from their phylogenetic relatives. Since only some of the properties have been examined so far, further characterizations are still needed to describe these strains. They should include the pH range of growth and optimum pH, utilization of nutrients (sugars, sugar alcohols, amino acids) , presence of some enzymes, quinone composition, fatty acids composition and the DNA G+C content.

The attempts of PCR amplification of *puf* genes in the most isolates failed in this study. Although the PCR primers were universal primers, the primers may not fit to the

puf sequences of the isolates. Future works are needed to clarify the *puf* gene sequences of the new isolates.

This study demonstrated some characteristics of novel aerobic anoxygenic photosynthetic bacteria isolated from the river biofilm. Further characterization of the isolates is still needed to describe them as novel species. It will also help to understand the roles of these bacteria in ecosystem of rivers.

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Table III-1. Cell size and shape of the isolates

Strain	Width(μm)	Length(μm)	Shape
S08	0.7-0.8	1.1-1.5	rods
W09	0.6-0.7	0.9-1.2	rods
W19	0.5-0.6	1.0-1.2	rods, a single cell or 2-4cells connected
W32	0.5-0.6	1.0-1.2	rods
S19	0.5-0.6	1.2-1.5	rods
W17	0.5-0.6	1.0-1.3	rods
S16	0.5-0.7	1.2-1.5	rods
W35	0.8-1.1	0.8-1.5	ovoid rods

Table III-2. *In vivo* absorption peaks of the isolates (sonicated)

Strain	Media	Absorption peaks of BChl (nm)					LH proteins	Other absorption peaks (nm)			
S08	PE			798		866	LH Ⅰ				
W09	PE			799		871	LH Ⅰ				
	1/5PE			803		871					
W19	1/5PE			804		866	LHI, LHII				
W32	PE			802		868	LH Ⅰ				
	1/5PE					865					
S19	PE			801	839	867	LHI, LHII	431	457	489	
	1/5PE	369	582	801	838				457	489	
W17	PE				824	868	LHI, LHII	432	455	488	
	1/5PE	368	580	804	822	868			455	489	
S16	PE		583	800	853	*	LHI, LHII	377	421	457	490
W35	PE					871	LH I	360			
	1/5PE					871		361			

*S16 has an absorption shoulder around 860 nm

Table III-3. Growth temperature of the isolates

Strain	Temperature(℃)							
	4	10	15	20	25	30	35	40
S08	-	+	+	+	+	++	+	- -
W09	-	+	+	+	+	++	+	- -
W19	-	+	+	+	++	++	+	- -
W32	-	+	+	+	+	++	+	- -
S19	-	+	+	+	+	++	- -	- -
W17	-	+	+	+	+	++	- -	- -
S16	-	+	+	+	+	++	+	- -
W35	-	+	+	+	+	++	+	- -

Inoculated agar plates were incubated in the dark for 14 days under temperatures shown in the table.
Each 2 agar plates of PEA and 1/5 PEA media were used.
+: Growth was observed in both or either medium plates
++: Optimum growth was observed
-: Growth was not observed in the temperature, but after transfer to 30°C, growth was observed.
- -: Growth was not observed. Even after transfer to 30°C, growth was not observed.

After 14 day incubation, plates on which bacterial growth were not observed were transferred into an incubator at 30^{O}C.
All plates transferred from 4^{O}C showed immediate bacterial growth, but all plates from 35^{O}C and 40^{O}C did not grow.

All strains did not grow at 0^{O}C, 45^{O}C or 50^{O}C.
At 0^{O}C, growth were not observed, but after transfer to 30°C, growth were observed.
At 45^{O}C or 50^{O}C, growth were not observed and even after transfer to 30°C, growth were not observed neither.
(Details are listed in Table III-S1)

Table III-4. Temperature range for growth of described aerobic photosynthetic bacteria

Species	Temperature range for growth (℃)	Optimum temperature (℃)	Habitat	Reference
Roseomonas aestualii	20-40	30	Estuarine tidal waters, India	16
Aquincola tertiaricarbonis	4-40	30	Ground water, Germany	12
Staleya guttiformis	4-32	12-20	Hypersaline lake, Antarctica	11
Porphyrobacter neustonensis	10-37	28-30	Freshwater subtropical pond	5
Roseovarius tolerans	3-43.5	8.5-33.5	Hypersaline lake, Antarctica	10
Roseateles depolymerans	5-43	35	River in Ibaraki, Japan	20

Table III-5. Characteristics of the isolate S08, K3 with Roseomonas species
Data are from Jiang *et al.* (2006) (Reference 7), Ramana *et al.* (2010) (Reference 16) and this study (including Chapter IV).

	S08	K3**	*Roseomonas lacus*	*Roseomonas aestualii**
Cell size (width)	0.7-0.8 μm	0.7-0.8 μm	0.5-0.8 μm	0.6-1.0μm
Cell size (length)	1.1-1.5 μm	1.1-1.2 μm	0.8-1.5 μm	1.5-2.0 μm
Absorption peaks	798 nm and 866 nm	not determined	not reported	452 nm and 974 nm
Light harvesting protein	LH I	not determined	not reported	not determined
Growth temperqture	10°C to 35°C	10°C to 35°C	15°C to 40°C	20ºC to 40ºC
Optimum temperature	30°C	30°C	30°C	30°C
Colony color	pink-orange	pink-orange	colorless to pale-pink	orange
NaCl torelance	not determined	not determined	not determined	0% to 1%
Habitat	river biofilm in Japan	river biofilm in Japan	freshwater lake sediment in China	estuarine tidal waters in India
16S rRNA identiry	100.0%		98.1% to S08	93.4% to S08

*Spectral analysis of the methanol-acetone extract indicates the presence of bacteriochlorophyll.

**Isolation of strain K3 was documented in Chapter IV.

Table III-6. Characteristics of the isolate W09, W19 and W32 with *Tabrizicola aquatica*
Data are from Tarhriz *et al.* (2013) (Reference 22) and this study.

	W09	W19	W32	*Tabrizicola aquatica**
Cell size (width)	0.6-0.7 μm	0.5-0.6 μm	0.5-0.6 μm	0.9μm
Cell size (length)	0.9-1.5 μm	1.0-1.2 μm	1.0-1.2 μm	1.3-3 μm
Absorption peaks	800 nm and 871 nm	804 nm and 866 nm	802 nm and 865 nm	not reported
Light harvesting protein	LH I	LH I and LH II	LH I	not reported
Growth temperqture	10°C to 35°C	10°C to 35°C	10°C to 35°C	15°C to 55°C
Optimum temperature	30°C	25°C to 30°C	30°C	40°C to 45°C
Colony color	pink beige	pink beige	pink beige	colorless or cream colored
NaCl torelance	not determined	not determined	not determined	0% to 3%
Habitat	river biofilm in Japan	river biofilm in Japan	river biofilm in Japan	freshwater lake in Iran
16S rRNA identiry	100.0%	98.8% to W09	99.2% to W09	98.4% to W09, 98.8% to W19 and 99.2% to W32

*The presence of photosynthesis genes pufL and pufM was not shown and photosynthesis pigments were not formed (reference 21).

Table III-7. Characteristics of the isolateS19, W17 and S16 with *Polymorphobacter multimanifer* and *Sandarakinorhabdus limnophila*
Data are from Fukuda et al.(2014), Gich and Overmann (2006) (Reference 2, 3) and this study.

	S19	W17	S16	*Polymorphobacter multimanifer**	*Sandarakinorhabdus limnophila*
Cell size (width)	0.5-0.6 µm	0.5-0.6 µm	0.5-0.7 µm	pleomorphic	0.25-0.47µm
Cell size (length)	1.2-1,5 µm	1.0-1,3 µm	1.2-1,5 µm	pleomorphic	0.61-0.99 µm
Absorption peaks	801nm, 838nm, 867nm	804nm,822nm,868nm	800 nm and 865 nm	not reported	800, 837 and 865 nm
Light harvesting protein	LH I and LH II	LH I and LH II	LH I and LH II	not reported	LH I and LH II
Growth temperqture	10°C to 30°C	10°C to 30°C	10°C to 35°C	-4°C to 30°C	not described
Optimum temperature	30°C	30°C	30°C	25°C	not described
Colony color	brown	brown	red-brown	brown	orange-red
Habitat	river biofilm in Japan	river biofilm in Japan	river biofilm in Japan	crack of antarctic white rock	mesotrophic freshwater lake in Germany
16S rRNA identiry	100.00%	99.93% to S19	95.24% to S19	98.03% to S19	95.24% to S19 95.16% to S19 98.59% to S16

*Oligotrophic bacterium. No growth was observed in 0.75 x LB medium, grew in 0.25 x LB/ASW medium.

Table III-8. Characteristics of the isolate W35 with *Aquincola tertiaricarbonis*
Data are from Lechner *et al.* (2007) (Reference 12),
Rohwerder et al. (2013) (Reference 17) and this study.

	W35	*Aquincola tertiaricarbonis**
Cell size (width)	0.8-1.1 μm	0.8-1 μm
Cell size (length)	0.8-1,5 μm	1.2-2 μm
Absorption peaks	871 nm	not described
Light harvesting protein	LH I	not described
Growth temperqture	10°C to 35°C	4°C to 40°C
Optimum temperature	30°C	30°C
Colony color	beige	white
Habitat	river biofilm in Japan	aquifer in Germany
16S rRNA identiry	100.0%	97.3%

*This species produce bacteriochlorophyll only under extremely low nutrient conditions

Table III-S1. Growth temperature of the isolates (detail)

Strain	Media	Temperature(℃)										
		0	4	10	15	20	25	30	35	40	45	50
S08	PEA	-	-	+	+	+	+	++	+	- -	- -	- -
	1/10PEA	-	-	+	+	+	+	++	+	- -	- -	- -
W09	PEA	-	-	+	+	+	+	++	-	- -	- -	- -
	1/10PEA	-	-	+-	+	+	+	++	+	- -	- -	- -
W19	PEA	-	-	-	+	+	++	+	+*	- -	- -	- -
	1/10PEA	-	-	+-	+	+	++	++	+	- -	- -	- -
W32	PEA	-	-	+-	+	+	+	++	+	- -	- -	- -
	1/10PEA	-	-	+-	+	+	+	++	+	- -	- -	- -
S19	PEA	-	-	+	+	+	+	++	- -	- -	- -	- -
	1/10PEA	-	-	+	+	+	+	++	- -	- -	- -	- -
W17	PEA	-	-	+	+	+	+	++	- -	- -	- -	- -
	1/10PEA	- -	- -	+	+	+	+	++	- -	- -	- -	- -
S16	PEA	-	-	+	+	+	+	++	+	- -	- -	- -
	1/10PEA	-	-	+	+	+	+	++	+	- -	- -	- -
W35	PEA	-	-	+	+	+	+	++	+	- -	- -	- -
	1/10PEA	- -	-	+	+	+	+	++	+	- -	- -	- -

Cultivations were conducted in the dark for 14 days using each 2 agar plates of PEA and 1/10PEA media.

+ : Growth was shown.

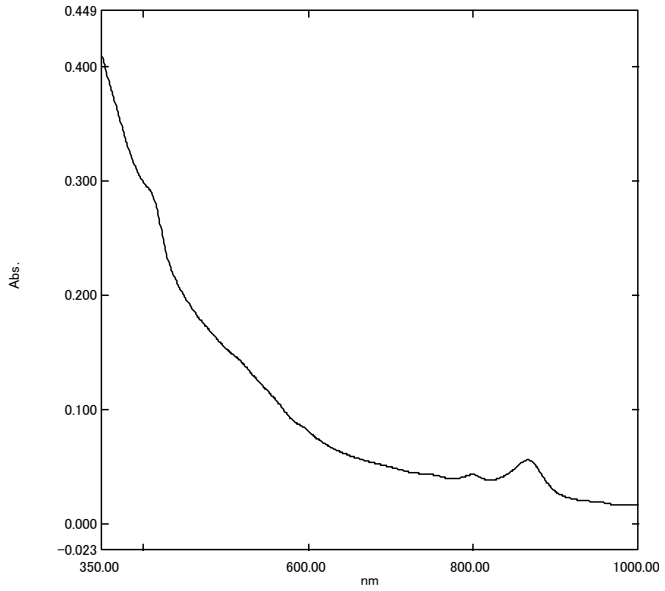
++ : Optimum growth was shown in the temperature.

- : Growth was not shown in the temperature and after transfer to 30 ℃, growth was shown.

- -: Growth was not shown. (even after transfer to 30 degree Celsius, growth was not shown.)

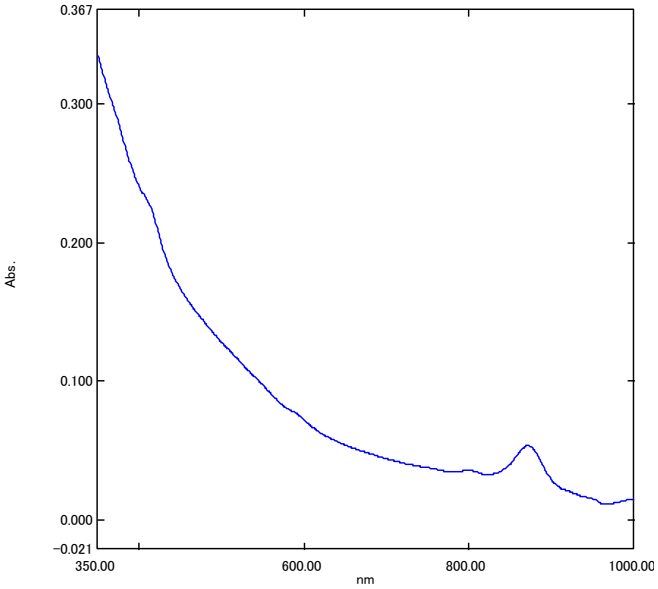
+* : Only one colony was grown.

+- : Little growth was shown.

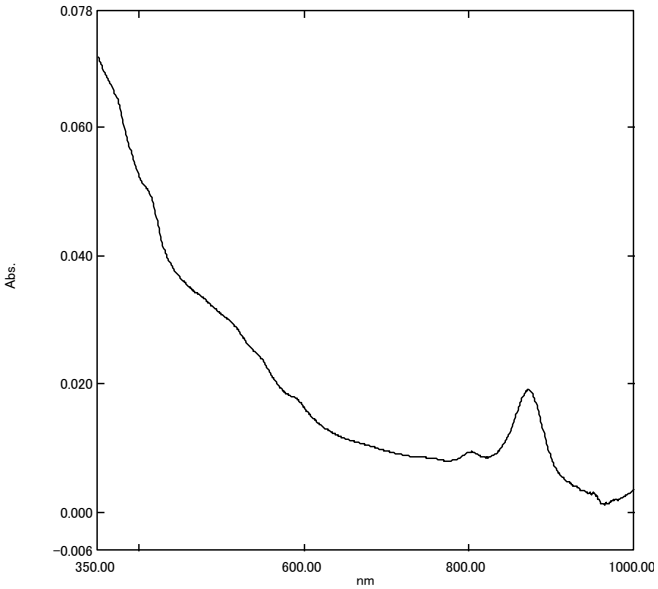


(a) S08-PE

Fig. III-S1. *In vivo* spectra of the isolates (sonicated supernatant)

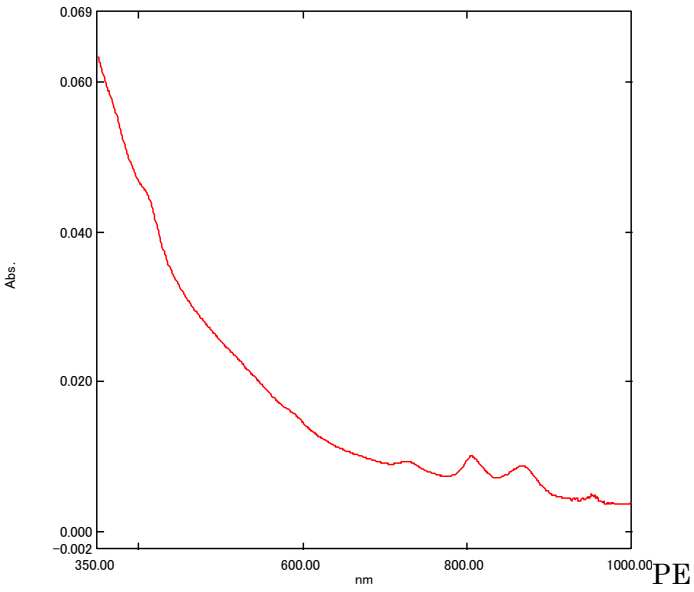


(b) W09-PE



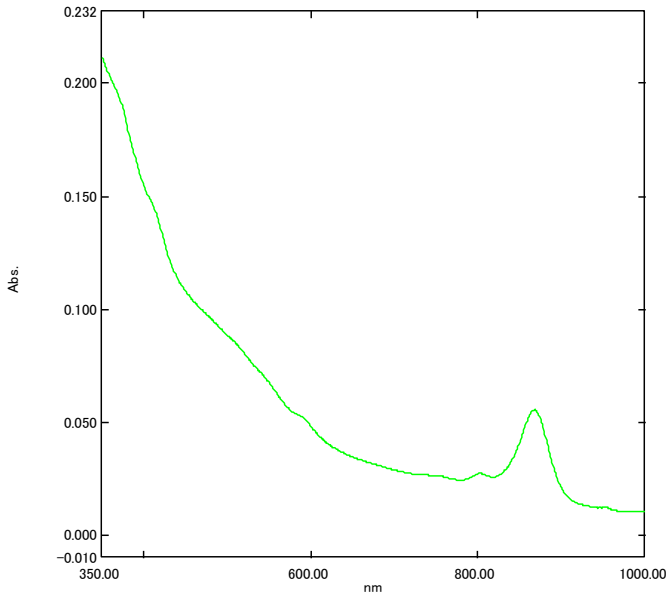
(c) W09-1/5PE

Fig. III-S1. *In vivo* spectra of the isolates (sonicated supernatant) (continued)

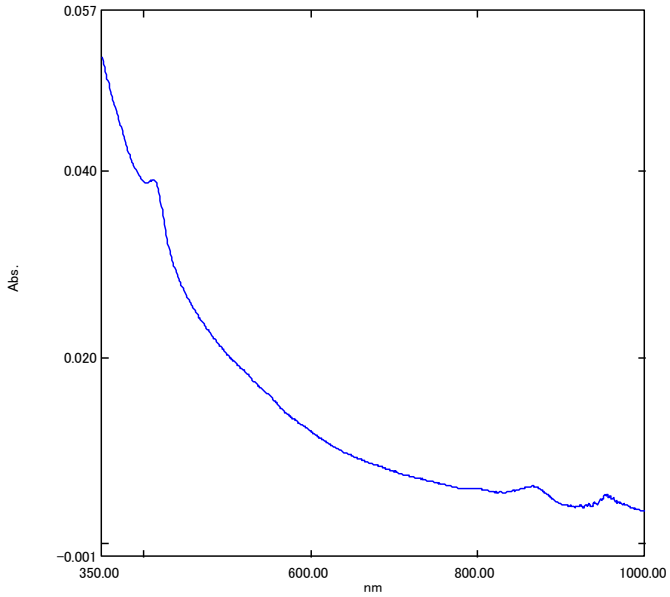


(d) W19-1/5PE

Fig. III-S1. *In vivo* spectra of the isolates (sonicated supernatant) (continued)

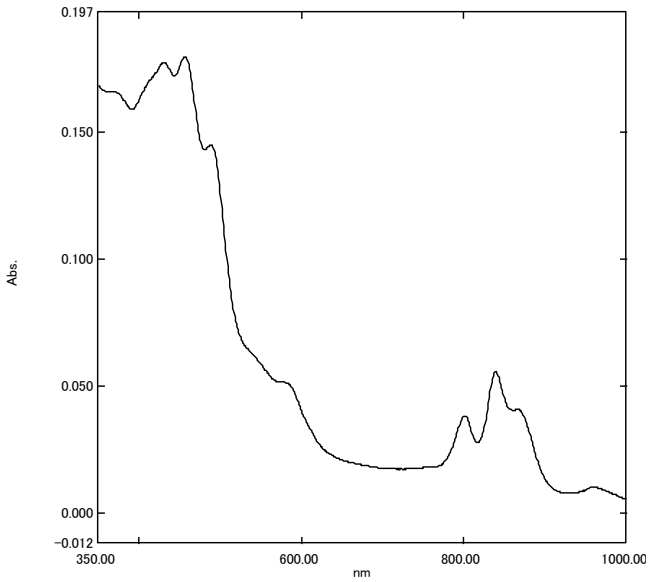


(e) W32-PE

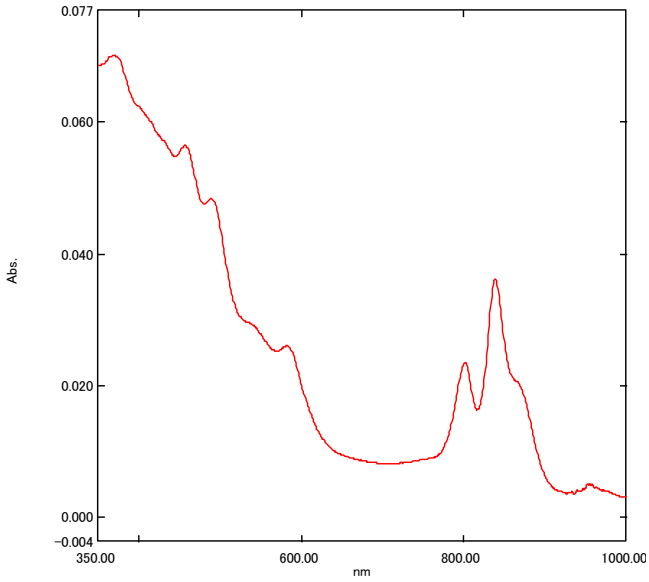


(f) W32-1/5PE

Fig. III-S1. *In vivo* spectra of the isolates (sonicated supernatant) (continued)

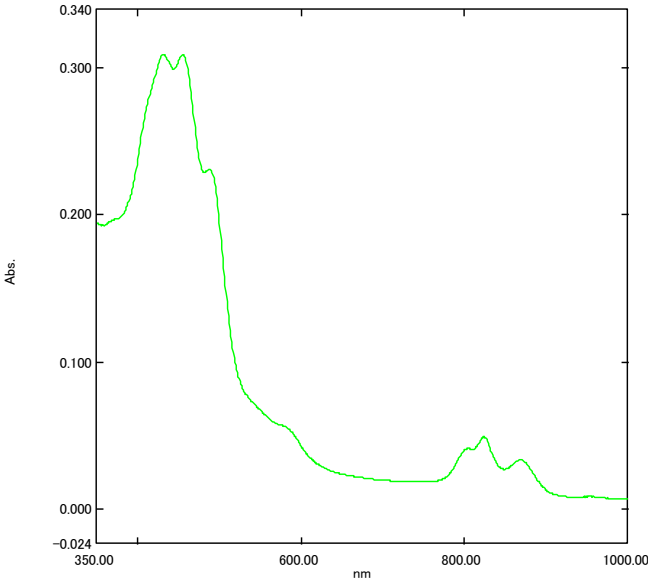


(g) S19-PE

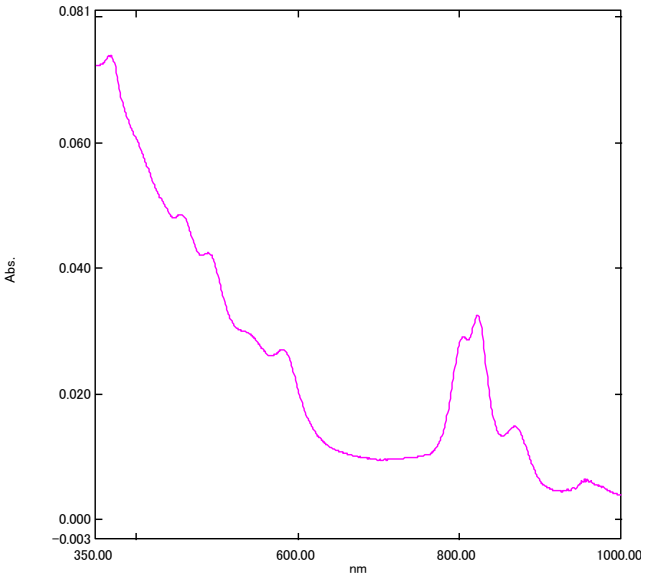


(h) S19-1/5PE

Fig. III-S1. *In vivo* spectra of the isolates (sonicated supernatant) (continued)

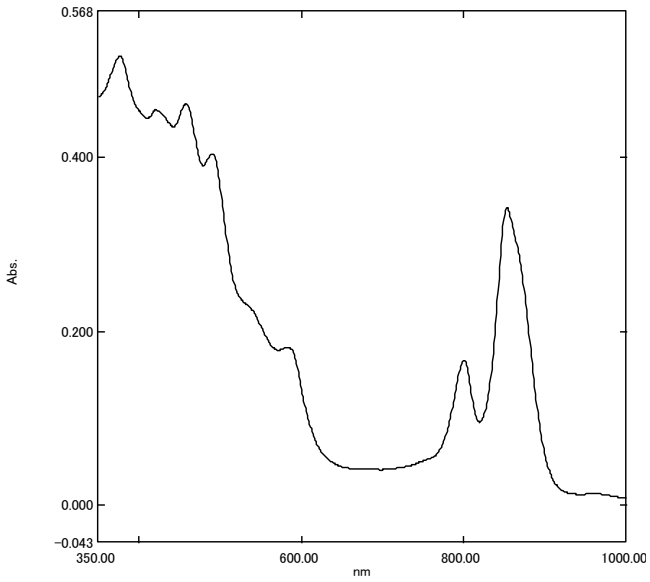


(i) W17-PE



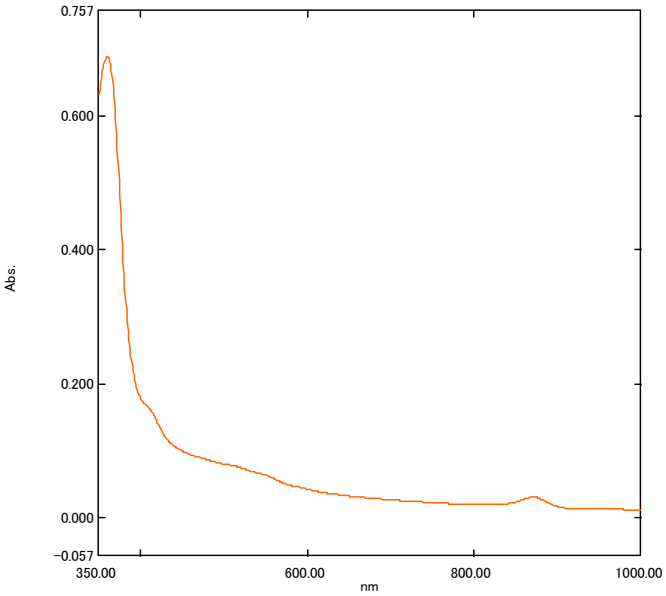
(j) W17-1/5PE

Fig. III-S1. *In vivo* spectra of the isolates (sonicated supernatant) (continued)

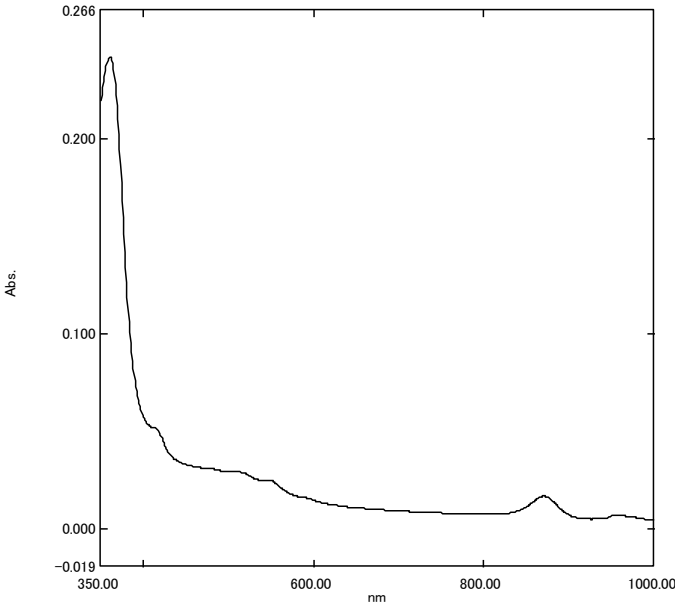


(k) S16-PE

Fig. III-S1. *In vivo* spectra of the isolates (sonicated supernatant) (continued)



(l) W35-PE



(m) W35-1/5PE

Fig. III-S1. *In vivo* spectra of the isolates (sonicated supernatant) (continued)

Using 500 ml shaking flasks, 1ml culture was inoculated into 100 ml medium and shaked 78 rpm at 30ºC in the darkness. Cells were cultivated for 7 days. About 30 ml culture was harvested and stored in -20ºC till measurement. Stored cells were suspended in 10 ml of 10 mM Tris-HCl buffer pH 8.0 and sonicated. The centrifuged supernatant was used for measurement of absorption spectra. Figures (a) to (m) are absorption spectrum of each isolate, for example, (m) W35-1/5PE indicates the isolate W35 cultivated in 1/5 PE medium.

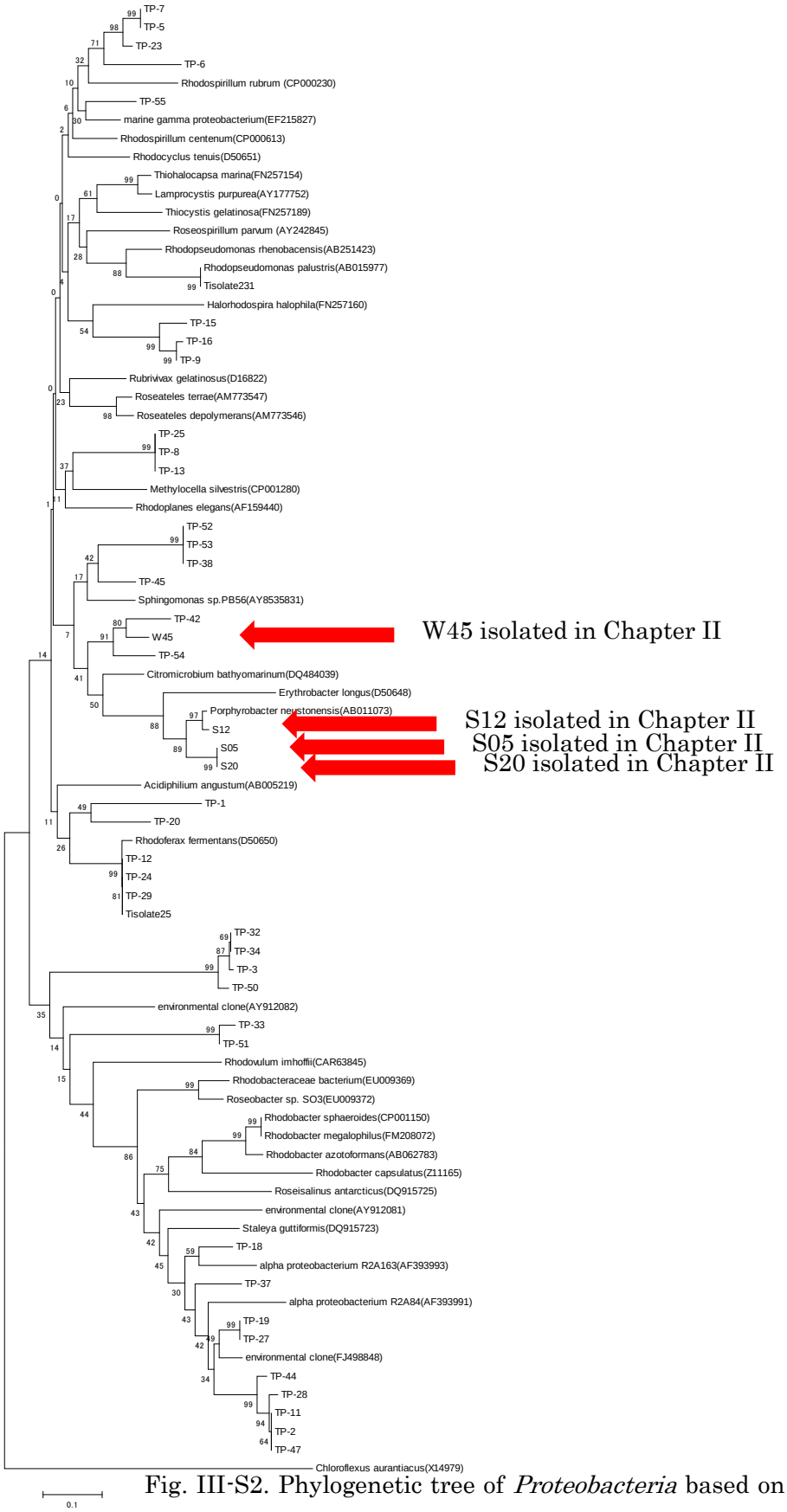


Fig. III-S2. Phylogenetic tree of *Proteobacteria* based on partial PufM amino acid sequences inferred from nucleotide sequences

Fig. III-S2. Phylogenetic tree of *Proteobacteria* based on partial PufM amino acid sequences inferred from nucleotide sequences of its gene. Neighbor-joining method was used. *Chloroflexus aurantiacus* was used as an outgroup. PufM amino acid sequence from aerobic anoxygenic photosynthetic isolate in Chapter II are indicated by the 'W' or 'S' prefix, those from environmental DNA in Chapter I are indicated by TP-1 to TP-55, and those from isolates in Chapter I are indicated by the 'Tisolate' prefix. Sequences from the database are represented with their respective accession numbers after bacterial names in parentheses. Bootstrap values are shown at intersection. Scale bar represents the number of substitutions per site.

CHAPTER IV

Phylogentic Diversity of Anoxygenic Photosynthetic Bacteria Isolated from Epilithic Biofilms in Tama River in Okutama-machi, Upstream of Ohme-city

Abstract

Phylogenetic diversity of anoxygenic photosynthetic bacteria in the river biofilm of Okutama-machi, 20 km upstream of Ohme-city, Tama River, was investigated using a cultivation method. Randomly selected 13 strains of aerobically grown bacteriochlorophyll-containing bacteria were isolated. The isolated strains were phylogenetically divided into 10 groups, as each group having 96% or more 16S rRNA sequence identities, belonging to the alpha-1 and alpha-4 subclasses of class *Alphaproteobacteria* and the class *Betaproteobacteria.* Among them 9 groups had 94.6-98.6% sequence similarities to known bacteria. Five groups showed similar phylogenetic positions to those isolated in Ohme-city but others had not been detected from Ohme-city. These results suggest that the two sites at Okutama-machi and Ohme-city have some common habitats as well as different habitats in the river biofilms for aerobic photosynthetic bacteria.

Introduction

In the ocean, aerobic anoxygenic photosynthetic bacteria have been recognized to account for a considerable fraction (11% of the marine bacterioplankton), and reported to have significant roles for the marine carbon cycling (4, 5). However, the presence or absence and diversity of aerobic anoxygenic photosynthetic bacteria in freshwater environments have been poorly understood.

From the river biofilm of Tama River, 28 strains of aerobic anoxygenic photosynthetic bacteria were isolated and phylogenetically divided into 8 groups belonging to alpha-1, alpha-3 and alpha-4 subclasses of *Alphaproteobacteria* and *Betaproteobacteria*. Among them, at least 4 novel lineages were recognized (Chapter II). It was, however, conducted at one sampling site in Ohme-city. In this chapter in order to know whether these isolates are commonly present in the biofilms of upper reaches of the same river, the isolation from another sampling site was performed.

In this chapter, using the same culture-dependent approaches as performed in Ohme-city in Chapter II, I investigated with Ms. Chizuru Kato, an undergraduate student of Biological Science Course, Tokyo Metropolitan University, the diversity of anoxygenic photosynthetic bacteria in the biofilm of the same river. The new sampling was done 20 km upstream of the sampling site in Chapter II, at Okutama-machi in Tama River, where environmental conditions such as river width and flow velocity are significantly different from those of the site in Ohme-city.

Materials and Methods

Sampling of epilithic biofilms

Submerged cobbles of about 15 to 20 cm in the longest length were collected from a streambed in a riffle located in a branch of the upper reaches of Tama River, Nishikawa, in Okutama-machi (35°49'07"N, 139°7'26"E) Tokyo, Japan (Fig. IV-1, Fig. IV-2). This site locates 20 km upstream of the site in Ohme-city, which was the sampling point of Chapter I and II. The riffle width and water depth at the sampling site were about 5 m and 0.1 m, respectively. The riverside was about 5 to 10 m and the stream was surrounded by forests. Therefore, the trees make shade to the stream especially in summer. In the upstream of the sampling point, there is no farmland and only a few people live in there. Therefore, the effects of human-derived materials to the sampling site should be very limited.

Biofilm of about 1 mm thickness was present on the cobbles. Three cobbles were taken from the streambed and a total 150 cm^2 area of the epilithic biofilm was scraped off from the top surface of each cobble using a sterilized toothbrush and suspended in 10 ml sterilized distilled water. Samples were kept cool in ice and brought to the laboratory as described in Chapter II.

Isolation of bacteriochlorophyll-containing aerobic bacteria

Isolation was carried out with a similar method to that in Chapter II. The suspended biofilm aggregates were dispersed using POLYTRON PT10/35 (KINEMATICA, Luzern, Switzerland) in a chilled bowl, and then diluted and directly spreaded onto agar plates. Agar plates of 1/10 PE medium (containing 0. 25 g of organic

compounds per litter, documented in Chapter II) supplemented with 1.5% (W/V) of agar were used for the isolation. Inoculated plates were cultivated aerobically at 30°C in the dark for 14 days. Red or pink colored colonies were randomly selected and transferred to new agar plates of 1/10 PE medium and cultivated aerobically at 30°C in the dark for a week. After the cultivation, the absorption spectra of colonies were determined using spectrophotometer V-630 (Jasco corporation, Tokyo, Japan) to detect bacteriochlorophyll which bound to the light-harvesting proteins. Colonies which showed absorbance maxima from 800 nm to 875 nm were transferred to new agar plates of 1/10 PEA medium. Transfer and cultivation were repeated more than twice to obtain pure cultures. These isolated strains were indicated by the ’K’ prefix, for example K3, in this chapter.

Sequencing of the 16S rRNA gene and phylogenetic analysis

Sequencing of the 16S rRNA genes and phylogenetic analyses were conducted using the same methods as described in Chapter II. Total genomic DNA of the isolates was extracted according to Noll et al. (7). DNA fragments of partial 16S rRNA gene were amplified using the primer sets 27F2/907R2 (6, 10). DNA sequences were determined with the BigDye v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and a DNA sequencer ABI3130xl (Applied Biosystems). To determine complete sequences, sequence primers 27F2, 515F, 517R and 907R2 were used and contig was made using ATGC program (GENETYX Ver. 12, GENETYX, Tokyo, Japan). Phylogenetic analysis was conducted using MEGA version 6 program (11).

Results

Bacteriochlorophyll-containing colonies from epilithic biofilm in Okutama-machi

Table IV-1 summarizes the number of isolated colonies from the river biofilms of Okutama-machi. We took three cobbles from the streambed and the results from each cobble are shown in Table IV-1. The cobbles are named as Okutama-machi-1, Okutama-machi-2 and Okutama-machi-3. 3.6×10^5 to 1.6×10^8 colonies per cm^2 of the biofilm were detected. About 36% of the total colonies were red or pink colored colonies. During the isolation process, 51 colored colonies were picked up and isolated. Within these, 13 colonies were confirmed to have bacteriochlorophyll.

Phylogenetic analysis of the isolates

16S rRNA gene sequences of 13 bacteriochlorophyll-containing isolates were determined. Figure IV-3 shows a neighbor-joining tree based on 16S rRNA gene sequences from 13 isolates obtained in this study together with those of isolates from Ohme-city (Chapter II) and from the database. A phylogenetic tree using the maximum-likelihood method (data not shown) showed tree topology mostly consistent with that in Fig. IV-3. The isolates were divided into 10 operational taxonomic units (OTUs) using a criterion of 96% sequence identity. These OTUs were belonging to the alpha-1 and alpha-4 subclasses of *Alphaproteobacteria* and *Betaproteobacteria*. The same OTU names designatd in Chapter II were used in this chapter.

K2, K8 and K9, belonging to OTU7 together with many Ohme-city isolates (Fig. IV-3), were closely related to an aerobic anoxygenic phototsynthetic bacterium, *Porphylobacter donghaensis*, with 99.4%-100% similarities. In contrast, the isolates in

other 9 OTUs were not closely related to the known bacteria (Fig. IV-3, Table IV-S1). These isolates showed 94.6-98.6% similarities to the closest relatives.

Isolate K3 belonged to the alpha-1 subclass of *Alphaproteobacteria*, and was most closely related to *Roseomonas lacus*, with 97.0% identity. Isolate K6, belonged to alpha-4 subclass of *Alphaproteobacteria* OTU 3. This isolate was closely related to the Ohme-city isolates involved in this OTU as well as to *Polymorphobacter multimanifer* with 98.4% similarity. Isolates K10 and K7 were distant from any known bacteria, 95.4% to *Sphingomonas mali* and 96.5% to *Sphingomonas desiccabilis,* respectively. Isolate K13 was related to *Sphingomonas jaspsi* with 98.6% identity and also related to the strain S10 isolated from the site Ohme-city.

Five strains K14, K11, K12, K4 and K5 belonged to *Betaproteobacteria*. They were classified into 4 distinct OTUs except K4 and K5 that were nearly identical. K14 and K12 were distant from any bacteria, and their nearest neighbor *Inhella fonticola* and *Leptothrix mobilis* with 96.8% and 94.6% similarities, respectively.

The neighbors of all the isolates except for OTU7 were non-photosynthetic bacteria.

Growth temperature

Growth temperature of the isolate K3 was compared with that of Ohme-city isolates (Table IV-2, Table IV-S2). K3 grew in the range of 10^{O}C to 35^{O}C but did not grow at 40^{O}C. After 14 days incubation at 40^{O}C, continuing another 14 days cultivation at 30^{O}C were conducted, but no growth was observed.

Cell size and morphology

Cells of strain K3 were short rod shaped, with 0.7 to 0.8 μm in width and 1.1 to 1.2 μm in length.

Discussion

In this Chapter, we isolated 13 strains of bacteriochlorophyll-containing bacteria from the river biofilm in Okutama-machi, at 20km upstream of Ohme-city (where the sampling site in Chapter II was located) in Tama River. Phylogenetic analysis based on 16S rRNA sequences of the isolates indicated 10 OTUs of anoxygenic photosynthetic bacteria belonging to the alpha-1 and alpha-4 subclasses of *Alphaproteobacteria* and *Betaproteobacteria* (Fig. IV-3). These results suggest that the large diversity of anoxygenic photosynthetic bacteria in the river biofilms is commonly observed both in the two distant sites of Tama River.

Nine OTUs within 10 OTUs of isolates showed 98.6% or less 16S rRNA gene sequence identities to any known bacteria, and especially isolates in 5 OTUs of the nine showed less than 97% identity to the known bacteria (OTU 1, 9, 10, 11, 13)(Fig. IV-3, Table IV-S1). These results suggest many novel lineages of anoxygenic photosynthetic bacteria are present in the river biofilm in Okutama-machi as well.

The Okutama-machi isolates shared 5 common OTUs, OTU1, OTU3, OTU5, OTU7 and OTU8 with the Ohme-city isolates. Among these, at least 3 OTUs, OTU1, OTU3 and OTU8, were novel lineages of aerobic anoxygenic photosynthetic bacteria first found in this river.

Extremely diverged strains were isolated from the site in Okutama-machi. As many as 10 OTUs of high diversity were obtained by a single sampling (13 strains) from very small area (3 cobbles taken within 10 m^2 area). Comparing these results with those of the Ohme-city isolates, more diverged lineages were obtained from a smaller

number of isolates in Okutama-machi, as the isolates from Ohme-city showed 5 OTUs of diversity in summer (15 strains) and winter (13 strains), respectively.

A characteristic of Okutama-machi isolates is that they contain many strains of *Betaproteobacteria* (Fig. IV-3). In Okutama-machi isolates, 5 strains within 13 strains (4 OTUs of 10 OTUs) belonged to *Betaproteobacteria*, whereas in Ohme-city isolates, only one strain within 28 strains (1 OTU of 8 OTUs) belonged to that. Anaerobic anoxygenic photosynthetic bacteria in *Betaproteobacteria* are all known to be freshwater bacteria and sulfide-rich water bodies do not provide favorable conditions for them (2). Aerobic anoxgenic photosynthetic bacteria in *Betaproteobacteria* may also have similar properties. The larger ratio of *Betaproteobacteria* in Okutama-machi isolates compared to Ohme-city isolates may be related to possible lower contents of sulfide in the river biofilm of the site in Okutama-machi.

Among the isolates obtained only from Okutama-machi samples, several OTUs are distantly related to any of the known bacteria (OTU9, OTU10, OTU11 and OTU13). Our trials of isolating anoxygenic photosynthetic bacteria have been only 3 times, Ohme-city winter, Ohme-city summer and Okutama-machi. However, we could isolate various anoxygneic photosynthetic bacteria including those of possible novel lineages. Either in Okutama-machi or in Ohme-city, new lineages of the bacteria different from those ever described were found in every isolation trial (Fig. IV-3). Therefore, a very large diversity of anoxygenic photosynthetic bacteria not recognized before must be present in the river biofilm.

The isolate K3 from Okutama-machi forms OTU1 with S08, which was isolated

from Ohme-city in the phylogenetic tree based on the 16S rRNA sequences (Fig. IV-3). Both isolates probably belong to the genus *Roseomonas*. However, from this genus, only one species *Roseomonas aestuarii* has been reported to have bacteriochlorophyll. The new isolates may be the second and the third aerobic anoxygenic photosynthetic species in this genus. Also, there is a possibility that some other species of *Roseomonas* produce photosynthetic pigments, although no information about bacteriochlorophyll production has been available.

OTU2 consists of only Ohme-city winter isolates; W07, W09, W19, W32 and W40. No Okutama-isolates were included in this OTU. As our sampling in Okutama-machi was conducted only in summer, sampling in winter may result in isolation of the Okutama-machi isolates consisting of this OTU.

OTU3 contained Okutama-machi isolate K6, as well as Ohme-city winter isolates W17, W04 and Ohme-city summer isolate S19. These strains contained in this OTU might generally appear in the biofilms of upper reaches of the river throughout the year.

Only two genera, *Roseateles* (9) and *Aquincola* (8), have been described as aerobic anoxygenic photosynthetic bacteria belonging to *Betaproteobacteria*. *Methyloversatilis universalis* also have been reported to have *puf* genes (3). Our 5 isolates of *Betaproteobacteria* from Okutama-machi formed 4 OTUs apart from each other. They were also distant from the Ohme-city isolate, the two described photosynthetic species and *Methyloversatilis universalis*. 16S rRNA sequence identities of the isolates in *Betapreoteobacteria* to the nearest neighbors were low (not exceed 98.6% identity). Therefore, our 5 isolates might be 4 novel species, or even new genera of the anoxygenic photosynthetic bacteria belonging to *Betaproteobacteria*.

Strain K3 did not grow at 40^{O}C (Table IV-2), showing a high temperature sensitivity as observed in many other isolates from Ohme-city. The temperature of the river water in the upper reaches of Tama river around Okutama-machi is low throughout the year (4.3^{O}C-19.3^{O}C at Shouwabasi, located in mainstream, 5 km upstream of meeting of the rivers from the sampling site; 4.6^{O}C-20.2^{O}C at Wadabashi, 17.5km downstream of it) (http://www.kankyo.metro.tokyo.jp/). High temperature sensitivity of K3, as well as many other isolates from Ohme-city, may be reflected the low temperature of the river water in the sampling sites.

Finally, this work showed that a large diversity of anoxygneic photosynthetic bacteria were present in the river biofilm of Okutama-machi. About a half of them resembled those observed in the isolates from Ohme-city. These lineages observed in common may be general in the upper reaches of Tama River. In addition, phylogenetically unique anoxygneic photosynthetic bacteria were also found in the river biofilms obtained from not only Ohme-city but also Okutama-machi. Because the significant amount of diversity was observed in both of the two sites, it is quite likely that phylogenetically diverse anoxygenic photosynthetic bacteria generally live in the epilithic biofilms of upper reaches of rivers.

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Table IV-1.

Numbers of total colonies, colored colonies and BChl-containing colonies

Sample	Total	Red or pink colored		
	CFU/cm^2	CFU/cm^2	%*	BChl**
Okutama-machi-1	1.2×10^6	3.9×10^5	33	2/21
Okutama-machi-2	1.6×10^8	3.6×10^7	23	8/19
Okutama-machi-3	3.6×10^5	1.9×10^5	53	3/10

CFU: Colony forming unit

%*: Colored CFU/Total CFU

BChl**: Bacteriochlorophyll-containing colonies/Colored colonies tested

Sampling was conducted on July 30, 2013

Table IV-2. Growth temperature of the isolates

Strain	Temperature(℃)							
	4	10	15	20	25	30	35	40
K3	-	+	+	+	+	++	+	- -
S08	-	+	+	+	+	++	+	- -
W09	-	+	+	+	+	++	+	- -
W19	-	+	+	+	++	++	+	- -
W32	-	+	+	+	+	++	+	- -
S19	-	+	+	+	+	++	- -	- -
W17	-	+	+	+	+	++	- -	- -
S16	-	+	+	+	+	++	+	- -
W35	-	+	+	+	+	++	+	- -

Inoculated agar plates were incubated in the dark for 14 days under temperatures shown in the table.
Each 2 agar plates of PEA and 1/5 PEA media were used.
+: Growth was observed in both or either medium plates
++: Optimum growth was observed
-: Growth was not observed in the temperature, but after transfer to 30°C, growth was observed.
- -: Growth was not observed. Even after transfer to 30°C, growth was not observed.

After 14 day incubation, plates on which bacterial growth were not observed were transferred into an incubator at 30^{O}C. All plates transferred from 4^{O}C showed immediate bacterial growth, but all plates from 35^{O}C and 40^{O}C did not grow.

Data of strains indicated by the ‘W’ or ‘S’ prefix are those from the study in Chapter III.

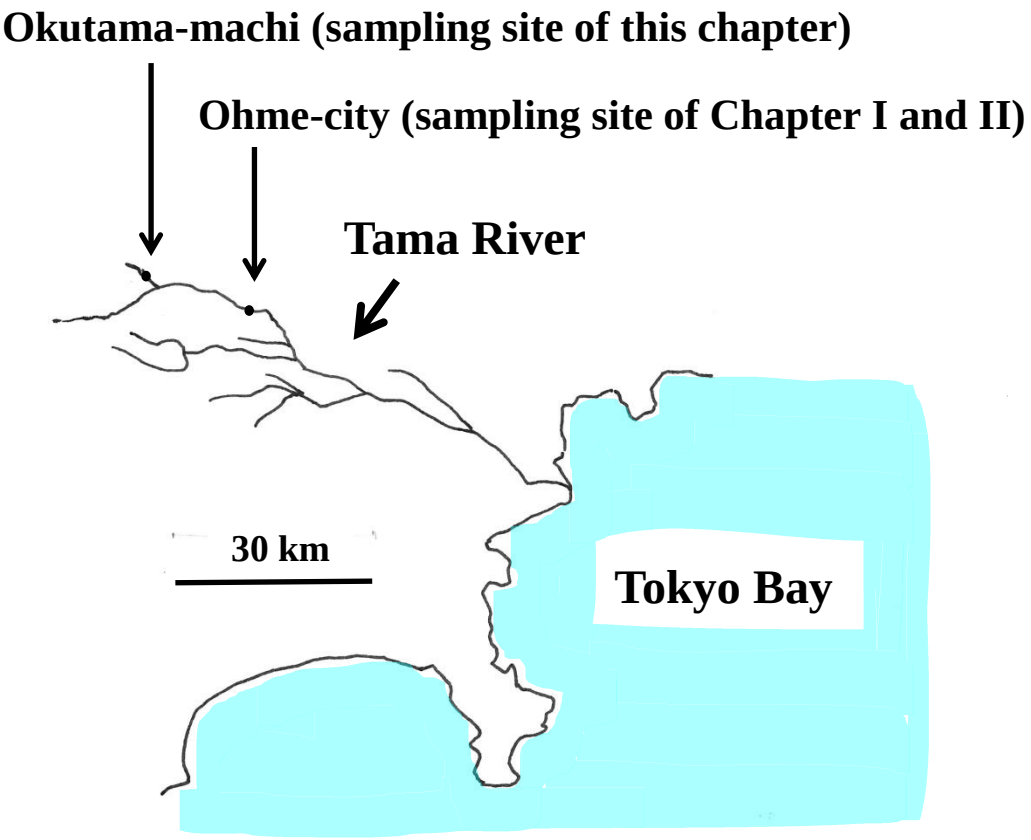

Fig. IV-1. Sampling site of this chapter.

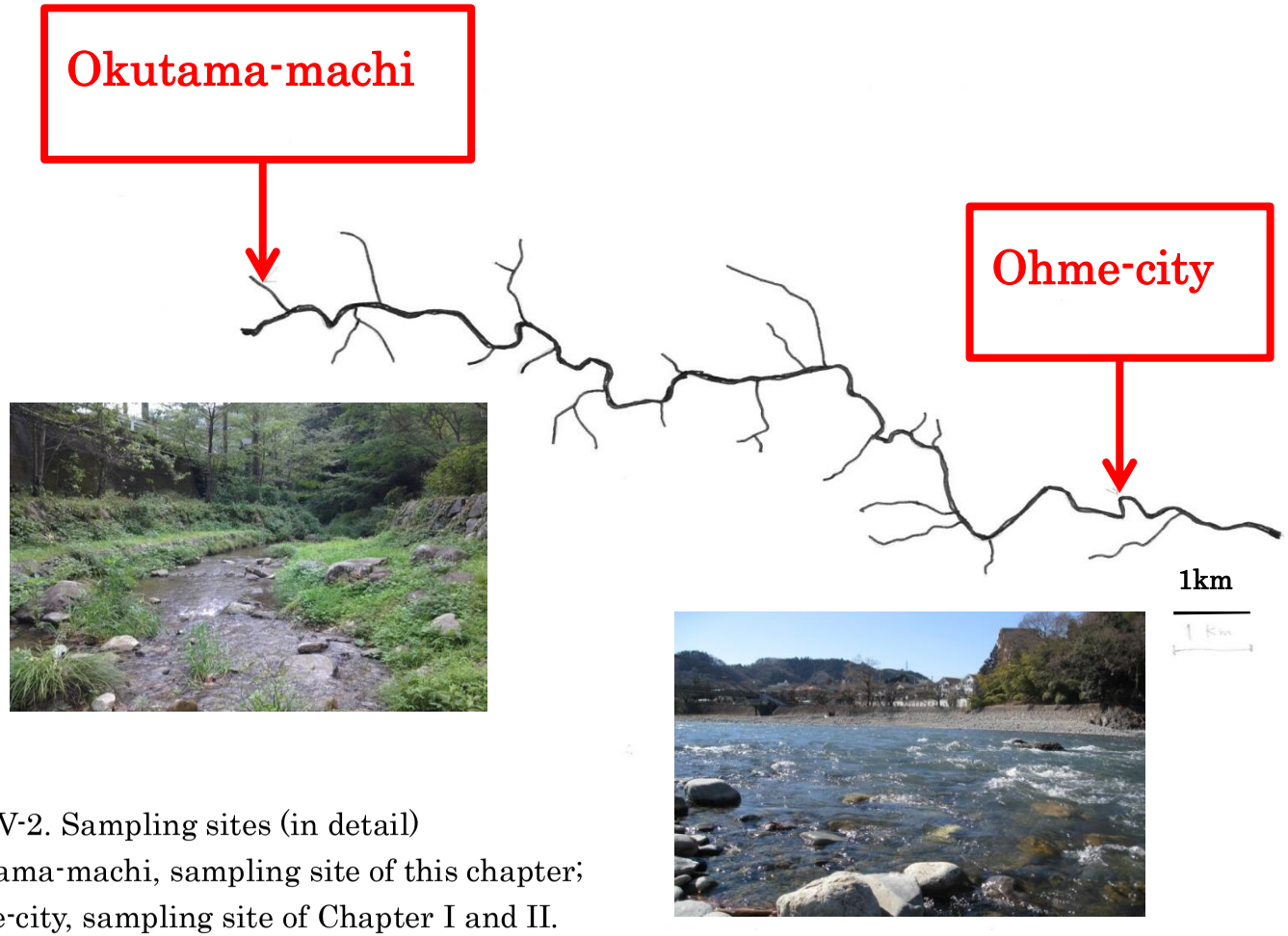

Fig. IV-2. Sampling sites (in detail)
Okutama-machi, sampling site of this chapter;
Ohme-city, sampling site of Chapter I and II.

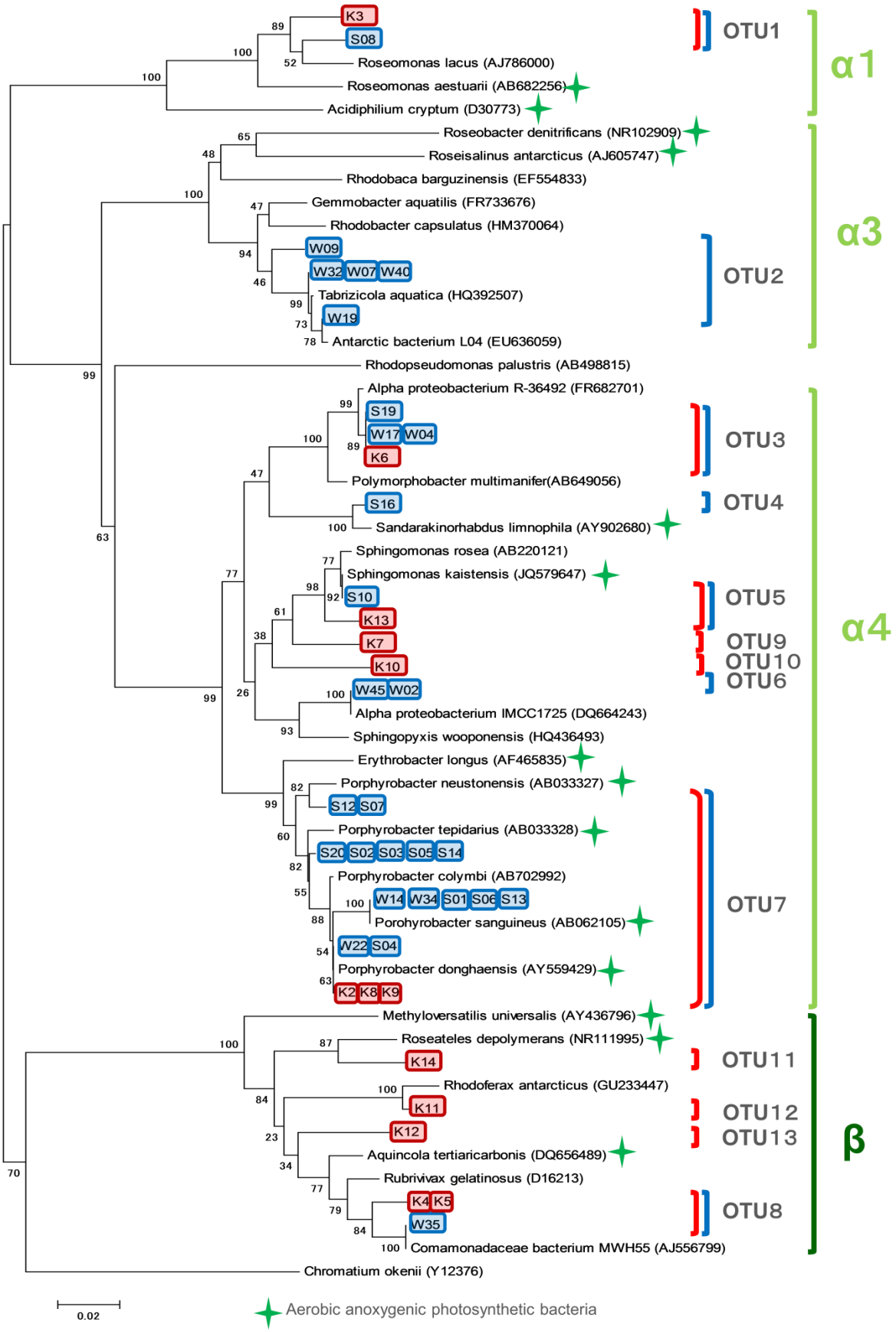
K3
S08
Roseomonas lacus (AJ786000)
Roseomonas aestuarii (AB682256)
Acidiphilium cryptum (D30773)
Roseobacter denitrificans (NR102909)
Roseisalinus antarcticus (AJ605747)
Rhodobaca barguzinensis (EF554833)
Gemmobacter aquatilis (FR733676)
Rhodobacter capsulatus (HM370064)
W09
W32 W07 W40
Tabrizicola aquatica (HQ392507)
W19
Antarctic bacterium L04 (EU636059)
Rhodopseudomonas palustris (AB498815)
Alpha proteobacterium R-36492 (FR682701)
S19
W17 W04
K6
Polymorphobacter multimanifer(AB649056)
S16
Sandarakinorhabdus limnophila (AY902680)
Sphingomonas rosea (AB220121)
Sphingomonas kaistensis (JQ579647)
S10
K13
K7
K10
W45 W02
Alpha proteobacterium IMCC1725 (DQ664243)
Sphingopyxis wooponensis (HQ436493)
Erythrobacter longus (AF465835)
Porphyrobacter neustonensis (AB033327)
S12 S07
Porphyrobacter tepidarius (AB033328)
S20 S02 S03 S05 S14
Porphyrobacter colymbi (AB702992)
W14 W34 S01 S06 S13
Porohyrobacter sanguineus (AB062105)
W22 S04
Porphyrobacter donghaensis (AY559429)
K2 K8 K9
Methyloversatilis universalis (AY436796)
Roseateles depolymerans (NR111995)
K14
Rhodoferax antarcticus (GU233447)
K11
K12
Aquincola tertiaricarbonis (DQ656489)
Rubrivivax gelatinosus (D16213)
K4 K5
W35
Comamonadaceae bacterium MWH55 (AJ556799)
Chromatium okenii (Y12376)
OTU1
OTU2
OTU3
OTU4
OTU5
OTU9
OTU10
OTU6
OTU7
OTU11
OTU12
OTU13
OTU8
α1
α3
α4
β
0.02
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Fig. IV-3.

Fig. IV-3. Phylogenetic tree of *Proteobacteria* based on partial 16S rRNA gene sequences. 16S rRNA gene sequences of the isolates obtained from Okutama-machi are indicated by the ‘K’ prefix accompanied with red boxes. Those obtained from Ohme-city on February and July are indicated by the ‘W’ and ‘S’ prefixes, respectively accompanied with blue boxes. Sequences from the database are represented with their respective accession numbers after bacterial name in parentheses. OTUs are indicated to the right of the tree. Alpha-1, alpha-3, alpha-4 subclass and beta class of *Proteobacteria* are also indicated by α-1, α-2, α-3 and β, respectively to the right of the tree. Bootstrap values are indicated at branching point. Scale bar represents the number of substitution per site.

Table IV-S1. Phylogenetic relatedness of Okutama-machi isolates to the described species.

Strain	Nearest neighbor		
	Accession no.		Similarity** (%)
K2	AY559429	Porphyrobacter donghaensis*	792/792 (100%)
K3	AM691116	Roseomonas lacus	737/760 (97.0%)
K4	FM886840	Leptothrix ginsengisoli	831/843 (98.6%)
K5	FM886840	Leptothrix ginsengisoli	730/742 (98.4%)
K6	AB649056	Polymorphobacter multimanifer	857/871 (98.4%)
K7	AJ871435	Sphingomonas desiccabilis	634/657 (96.5%)
K8	AY559429	Porphyrobacter donghaensis*	628/630 (99.7%)
K9	AY559429	Porphyrobacter donghaensis*	811/816 (99.4%)
K10	AB649021	Sphingomonas mali	771/808 (95.4%)
K11	FJ755906	Rhodoferax saidenbachensis	564/574 (98.3%)
K12	JF496490	Leptothrix mobilis	526/556 (94.6%)
K13	AB681706	Sphingomonas jaspsi	758/769 (98.6%)
K14	HM013811	Inhella fonticola	611/631 (96.8%)

* Aerobic anoxygenic photosynthetic bacteria.

**Similarity of 16S rRNA gene sequences.

Table IV-S2. Growth temperature of the isolates (detail)

Strain	Media	Temperature(℃)										
		0	4	10	15	20	25	30	35	40	45	50
K3	PEA	-	-	+-	+-	+	+	++	+	- -	- -	- -
	1/10PEA	-	-	+-	+	+	+	++	+	- -	- -	- -
S08	PEA	-	-	+	+	+	+	++	+	- -	- -	- -
	1/10PEA	-	-	+	+	+	+	++	+	- -	- -	- -
W09	PEA	-	-	+	+	+	+	++	-	- -	- -	- -
	1/10PEA	-	-	+-	+	+	+	++	+	- -	- -	- -
W19	PEA	-	-	-	+	+	++	+	+*	- -	- -	- -
	1/10PEA	-	-	+-	+	+	++	++	+	- -	- -	- -
W32	PEA	-	-	+-	+	+	+	++	+	- -	- -	- -
	1/10PEA	-	-	+-	+	+	+	++	+	- -	- -	- -
S19	PEA	-	-	+	+	+	+	++	- -	- -	- -	- -
	1/10PEA	-	-	+	+	+	+	++	- -	- -	- -	- -
W17	PEA	-	-	+	+	+	+	++	- -	- -	- -	- -
	1/10PEA	- -	- -	+	+	+	+	++	- -	- -	- -	- -
S16	PEA	-	-	+	+	+	+	++	+	- -	- -	- -
	1/10PEA	-	-	+	+	+	+	++	+	- -	- -	- -
W35	PEA	-	-	+	+	+	+	++	+	- -	- -	- -
	1/10PEA	- -	-	+	+	+	+	++	+	- -	- -	- -

Cultivations were conducted in the dark for 14 days using each 2 agar plates of PEA and 1/10PEA media.

+ : Growth was shown.

++ : Optimum growth was shown in the temperature.

- : Growth was not shown in the temperature and after transfer to 30 °C, growth was shown.

- -: Growth was not shown. (even after transfer to 30 degree Celsius, growth was not shown.)

+* : Only one colony was grown.

+- : Little growth was shown.

Data of strains indicated by the ‘W’ or ‘S’ prefix are those from the study in Chapter III.

Concluding Remarks

Aerobic anoxygenic photosynthetic bacteria have been found in various environments, but their distribution and diversity in natural environments have not been sufficiently studied except for ocean environments.

In this study, I found phylogenetically divergent aerobic anoxygenic photosynthetic bacteria in biofilms of the upper reaches of Tama River. Twenty eight strains of aerobic anoxygneic photosynthetic bacteria were isolated from a sampling site in Ohme-city. They were divided into 8 OTUs, having 96% to 100% 16S rRNA sequence identities within each OTU.

Additional sampling was conducted at Okutama-machi, 20 km upstream of the river at Ohme-city. Thirteen strains of aerobically grown bacteriochlorophyll-containing bacteria were isolated and divided to 10 OTUs, of which 5 OTUs were common with those of Ohme-city.

The isolates obtained in this study belonged to the alpha-1, alpha-3 and alpha-4 subclass of the class *Alphaproteobacteria* and the class *Betaproteobacteria*. They were widely distributed in the taxa in which aerobic anoxygneic photosynthetic bacteria have been known. So far aerobic anoxygneic photosynthetic bacteria have been mainly known in the class *Alphaproteobacteria*, and some freshwater species were also known in the class *Betaproteobacteria*. (Marine species were known in the class *Gammaproteobacteria*.)

Physiological examinations were also conducted in this study. Two strains grew in a low nutrient medium containing 0.5 g of organic compounds per liter but did not grow in a high-nutrient medium containing 2.5 g of organic compounds per liter. This may

reflect the environmental conditions where the isolates inhabited. But the other isolates grew both in the low- and high-nutrient media although some differences were observed in the relative growth rate between the low- and high-nutrients. The diversity may be because the river biofilms have various microniches for aerobic anoxygenic photosynthetic bacteria.

The studied isolates showed remarkable temperature dependence. They did not grow at a moderately high temperature, 40^{O}C. Some strains did not grow even at 35^{O}C. These upper limits are lower than those of most aerobic anoxygenic photosynthetic bacteria. The inability of growth in moderately high temperature may reflect the low temperature of the river water throughout the year.

Within the isolates, four novel lineages in Ohme-city isolates and another 4 novel lineages in Okutama-machi isolates were found. Until now about 60 species of aerobic anoxygenic photosynthetic bacteria have been described. In this study, novel lineages of over one tenth of known aerobic anoxygneic photosynthetic species were isolated and within these some were suggested to be novel genus even from only three sampling. The isolation of many novel lineages might be because river biofilms were the place where isolations of aerobic anoxygneic photosynthetic bacteria had not been conducted, and the biofilm environments were different from the sites isolations had been conducted.

In conclusion, the significant diversity of aerobic anoxygneic photosynthetic bacteria in the biofilm of the upper reaches of a river was revealed in this study. The upper reaches of the river have oligotrophic environments as in the ocean where aerobic anoxygenic photosynthetic bacteria have been reported to occupy a significant part of bacterioplankton.

Studies of aerobic anoxygenic photosynthetic bacteria in river environments are just beginning. To clarify the relationship between environmental conditions and various inhabited aerobic anoxygenic photosynthetic bacteria, more trials of isolations will be needed accompanying further physiological characterizations of the isolates.